Immune Responses against

Human Herpes virus 6

Mustafa Halawi

Student No. 200624270

CIMI, IGH
University of Liverpool,
Ronald Ross Building, West Derby Street,
Liverpool L69 7BE

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Declaration

All the work presented in this thesis is my own and any other work is appropriately referenced. The laboratory experiments have been carried out by me; in the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

Mustafa Halawi

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Abstract

Human herpes virus 6 (HHV6) infects the majority of individuals in childhood, followed by a lifelong asymptomatic latent infection. However, in immunosuppressed individuals reactivation of HHV6 can cause significant clinical pathology. Recent successes with adoptive T cell therapy against other viral infections, notably the human herpes viruses Epstein-Barr virus (EBV) and Human cytomegalovirus (HCMV), suggest that this may be a useful therapeutic approach for HHV6-driven disease in immunosuppressed individuals. However, very few studies have been carried out analysing the immune response to HHV6 in any detail. This thesis was aimed at characterising the CD8+ T cell response to HHV6 in a group of healthy individuals, with the aim of mapping and characterising novel CD8+ T cell epitopes.

Initial studies included four HHV6B antigens (U11, U39, U54 and U90), predicted to be immunogenic based on their HCMV homologues. Whole antigen peptide mixes (pepmixes) were used to stimulate peripheral blood mononuclear cells (PBMC) from healthy subjects. T cell responses were analysed by intracellular cytokine staining (ICS) after overnight stimulation and/or by interferon-γ (IFN-γ) ELISpot assay after 10 days of stimulation. For responses to U11 and U90, peptides libraries were used to map minimum CD8+ restricted epitopes. Further characterisation of HHV6B-specific T cells was carried out by identifying the HLA restriction elements and determining whether these T cells were capable of killing HHV6B-infected cells.

PBMC from 30 healthy donors were stimulated with pepmixes corresponding to HHV-6B antigens U11, U39, U54 and U90. A weak CD8+ response (0.02-0.2%) to U90 and U54 was observed in a number of donors.

Short-term *in-vitro* reactivations of PBMC (in 25 healthy donors) with HHV6B pepmixes followed by analysis of antigen and peptide specific response were performed by IFN-γ ELISpot assay. T cell responses to U54, U90, U11 and U39 were observed in 88%, 84%, 76% and 72% of the donors, respectively.

Subsequently, the breadth of epitope specificity within U90 and U11 was screened for 9 healthy donors; with successful identification of 10 CD8+ T cells specific (9-mer) epitopes within these antigens. Seven of them were within U90 antigens and three of them were within U11 antigens. Allelic association of the U90 epitopes were; VEESIKEIL - B40 (60), FESLLFPEL - B40 (60), NLITAAKNI - A2, ITAAKNIGI - A2, LNIDPSESI - A1, PSKSKKIKL - A29, NHCFINHFV - B39. Allelic association of the 2 U11 epitopes were LKTQRRHKF - B37 and GILDFGVKL - A2; the HLA association for FNAVYSQRV was not identified. CD8+ T cell populations specific to some of these epitopes were also able to kill HHV6B infected cells.

HHV6B T cells responses are detectable in healthy donors. Peptide specific responses against U11 and U90 have been mapped and characterised. These findings are relevant to the development of T cell mediated immunotherapy of HHV6-associated diseases.

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Abbreviations

AP	Alkaline phosphatase
APC	Allophycocyanin
APC	Antigen presenting cell
BFA	Brefeldin-A
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	5-(6) Carboxyfluorescein diacetate succinimidyl ester
CIHHV6	Chromosomally integrated HHV6
cm	Centimetre
CTL	Cytotoxic T cells
CO_2	Carbon dioxide
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E	Early
EBV	Epstein–Barr virus
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Centrifugal force
HCMV	Human cytomegalovirus
HD	Healthy donor
HHV	Human herpes virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IFN-γ	Interferon gamma
IE	Immediate-early
Ig	Immunoglobulin
IL	Interleukin
kb	Kilobase
L	Late
l	Litre
M	Molar

mA	Milliampere
mAb	Monoclonal antibody
mg	Milligram
MHC	Major Histocompatibility Complex
mCMV	Murine cytomegalovirus
ml	Millilitre
NK cell	Natural killer cell
OD	Optical density
ORF	Open Reading Frame
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-0.05%Tween20
PCho	Phosphorylcholine
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
pg	Picogram
PRR	Pathogen recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I Like Receptors
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SEM	Standard error of the mean
SFC	Spot forming cells
SSC	Side scatter
TBS	Tris buffered saline
TBS-T	Tris buffered saline-0.05%Tween20
TCR	T-cell receptor
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
VZV	Varicella Zoster virus
µg	Microgram
µl	Microlitre
µM	Micromolar
α	Alpha
β	Beta
γ	Gamma
δ	Delta

Chapter 1

1. Introduction

Human herpes viruses (HHVs) are ancient, widespread pathogens that have co-evolved with humans to achieve a fine host-pathogen balance of mutual co-existence. Unlike with many other viruses, primary infection with HHVs is followed by a persistent phase of infection. The work in this thesis concentrates on two such HHVs that fall in the beta-herpes virus sub-family. These are human herpes virus 6 (HHV6) and Cytomegalovirus (HCMV) as a control.

1.1 Herpes virus classification and basic biology

Herpes virus family is known as *Herpesviridae* (Table 1.1) and can infect a wide variety of living organisms. There are eight viruses of the *Herpesviridae* family that can infect human. The genome of the virus consists of a linear double-stranded DNA surrounded by a capsid, which is covered with a protein layer called the tegument, and an envelope composed of glycoproteins. These viruses are intracellular parasites and the host immune system can respond to unique antigens that are present in the virus glycoproteins. Herpes virus family members share several features beside genome and virion structure. They are capable of persisting within the body over long periods in a latent phase, enabling them to establish chronic infections. In latency, their genomes adopt an episomal form when they express a minimum of genes necessary for their maintenance thereby reducing recognition of the infected cell by the immune system (Whitley *et al.*, 2009).

Human herpes viruses are divided into three subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Mettenleiter *et al.*, 2009). The alpha

herpes viruses include herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) and varicella zoster virus (chicken pox). The gamma herpes viruses include Epstein Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV), also known as human herpes virus 8 (HHV-8) (Moss, 2007; Motta and Martins, 2008).

Finally, the beta herpes viruses include human cytomegalovirus (HCMV), human herpes virus 6 (HHV6) and human herpes virus 7 (HHV7). Beta herpes viruses have common growth characteristics, induces a cytopathology containing characteristic nuclear and cytoplasmic inclusions (Mocarski, 2002). They can remain latent in the secretory glands, lymphoreticular system and epithelial cells. HCMV is associated with severe congenital infections. HHV6 variant B (which is the common variant) and HHV7 are associated with a disease affecting young children called roseola or exanthema subitum (Arvin and Campadelli-Fiume, 2007).

Table 1.1 General information on the human herpes viruses family

	Alpha herpesviruses			Beta herpesviruses			Gamma herpesviruses	
Name	HSV-1	HSV-2	VZV	CMV	HHV-6 A&B	HHV-7	EBV	HHV-8
Primary infection	Gingivostomatitis	Genital sores	Chickenpox	Mononucleosis	Exanthema subitum (HHV-6B)	Exanthema subitum	Mononucleosis	–
Other diseases	Fever, blisters, encephalitis, retinitis	Encephalitis	Shingles, encephalitis	Retinitis, pneumonia	Hepatitis, encephalitis	–	cancers	Kaposi's sarcoma, cancers
Site of latency	Neurons	Neurons	Neurons	Monocytes, epithelia	Monocytes, T-cells	Monocytes, T-cells	B-cells	Uncertain
Prevalence	70%	50%	>99%	50%	>90%	>85%	>90%	<5%

HSV, herpes simplex virus; VZV, varicella zoster virus; HCMV, human cytomegalovirus; HHV, human herpes virus; EBV, Epstein–Barr virus.
Data on seroprevalence are valid for adult populations in western Europe and the USA regions.
'Other diseases' are typically found on reactivation of latent virus, or in immunocompromised individuals.

'Source: (Christensen, 2007)'

1.2 Human Herpes virus 6

Human herpes virus 6 (HHV6) was originally isolated in 1986 from the peripheral blood of six patients with lymphoproliferative disorders and/or infected with human immunodeficiency virus (HIV) (Salahuddin *et al.*, 1986). HHV6 and HCMV are closely related and belong to the β- herpes virus group (Efstathiou *et al.*, 1988). There are two known variants of HHV6 (A and B), of which variant B is the commoner (Dewhurst *et al.*, 1993). They have differences in DNA sequence, reaction to monoclonal antibodies, cell tropism and disease association (Pellett and Black, 1996). The GS or Uganda (U1102) like strain belongs to the variant A group, and the Zaire (Z29) or (HST) like strain belongs to the variant B group (Ablashi *et al.*, 1993). The HHV6A (strain GS) variant was the original isolate retrieved from an immunocompromised patient (Salahuddin *et al.*, 1986). The type B variant of HHV6 is associated with roseola (exanthem subitum) (Dewhurst *et al.*, 1993; Yamanishi *et al.*, 1988), while the pathogenesis of HHV6A is still unknown (Matsuura *et al.*, 2011; Isegawa *et al.*, 1999). Roseola is characterised by 3–5 days of fever with maculopapular rash. Moreover, HHV6 infection is reported to be associated with central nervous system infection, rejection of kidney transplant and hepatitis (Whitley *et al.*, 2009).

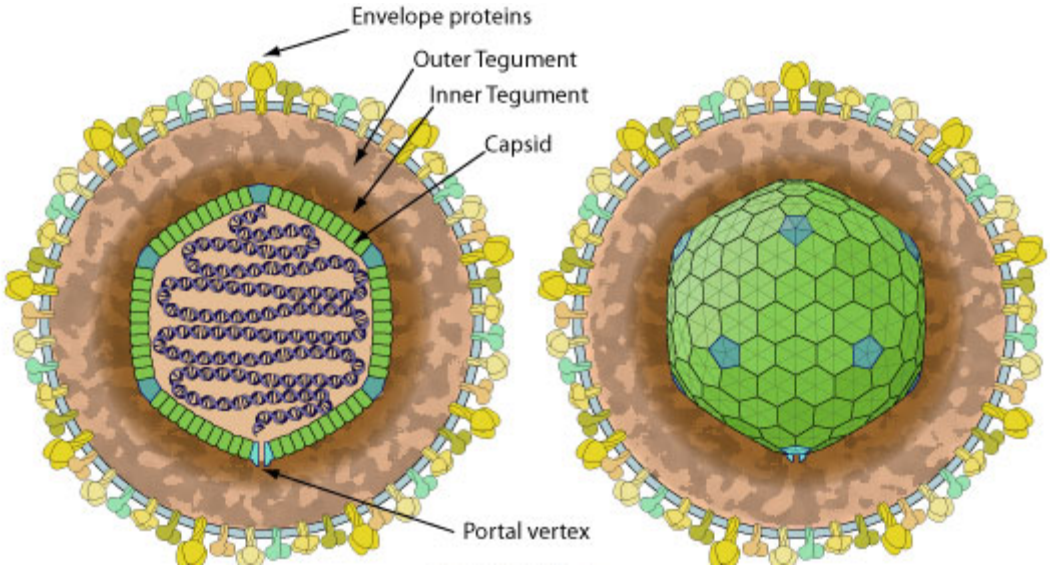


Figure 1.1 Human Herpes virus 6 (HHV6) structure

Figure showing HHV6 virion structure; which is enveloped, spherical to pleomorphic, approximately 200 nm in diameter. HHV6 has double linear DNA genomes covered by capsid proteins, consisting of 162 capsomers, encircled by an amorphous tegument. The lipid envelope is embedded with glycoprotein complexes. *(Hulo et al., 2011)*

1.3 Classification and basic biology of HHV6

Similar to other herpes viruses, the HHV6 virion is enveloped Figure (1.1), spherical, 200 nm in diameter, containing a single molecule of dsDNA which is 160 to 162kb in size (encoding about 100 proteins), with terminal direct repeats (DR_{left} and DR_{right}) of 8-9 kb in both flanks (De Bolle *et al.*, 2005; Tang and Mori, 2010). The genome contains 7 regions of genes conserved in all herpes viruses (Clark, 2000). There are three intermediate repeats (R1, R2 and R3) (Figure 1.2) interrupting a unique long (UL) region, at the immediate-early A (IE-A) region. The genomic structure of the unique long region is similar to that of human CMV and co-linear with that of HHV7. The genes in UL are designated as U1 to U100 and open reading frames (ORFs)

within the direct repeats are termed as DR1 to DR7. Genes coding for virion structure or enzymes needed for nucleotide metabolism and DNA replication, are seen to cluster in seven gene blocks. These features are common to all the herpes viruses. The HHV6B genome contains 119 ORFs from 97 genes, whereas the HHV6A genome has 110 (Dominguez *et al.*, 1999a), (Gompels *et al.*, 1995b). HHV6A and B variants share an overall nucleotide sequence identity of 90% (Dominguez *et al.*, 1999a). The IE-region of the genome is the most variable region between A and B variants (Chou and Marousek, 1994).

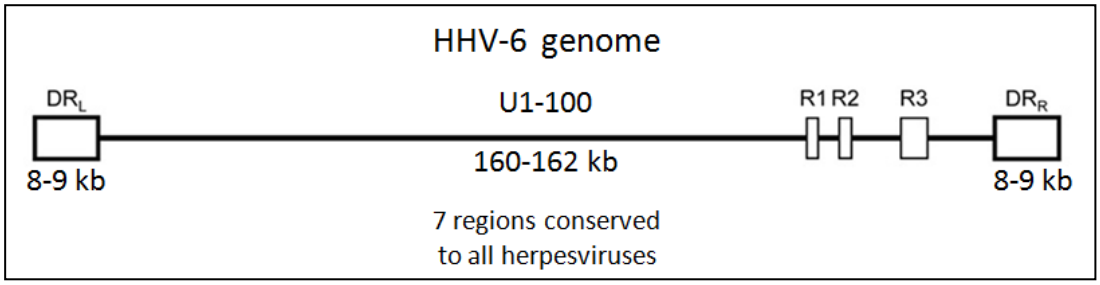


Figure 1.2 HHV6 genome

Figure showing the HHV6 genome structure, a double-stranded DNA of about 160 kbp, consisting of one long unique sequence (U) with identical direct repeats (DR_L and DR_R) in both flanks. The genome contains 7 regions conserved in all herpes viruses. *(Matsuura et al., 2011)*

Interaction with CD46 is required for the entrance of both the HHV6 (A and B) variants. All nucleated human cells express CD46, a type 1 glycoprotein (see Figure 1.3 below) (Santoro *et al.*, 1999), which acts as a regulator of complement activation. It has been shown that the gH-gL-gQ complex of HHV6 (the glycoproteins H, L and Q encoded by the HHV6 genes U48, U82 and U100), serves as the viral ligand for human CD46 (Mori *et al.*, 2003). HHV6 gB (encoded by U39) is also crucial for penetration of the virion into the cell (Takeda *et al.*, 1996) (Figure 1.3).

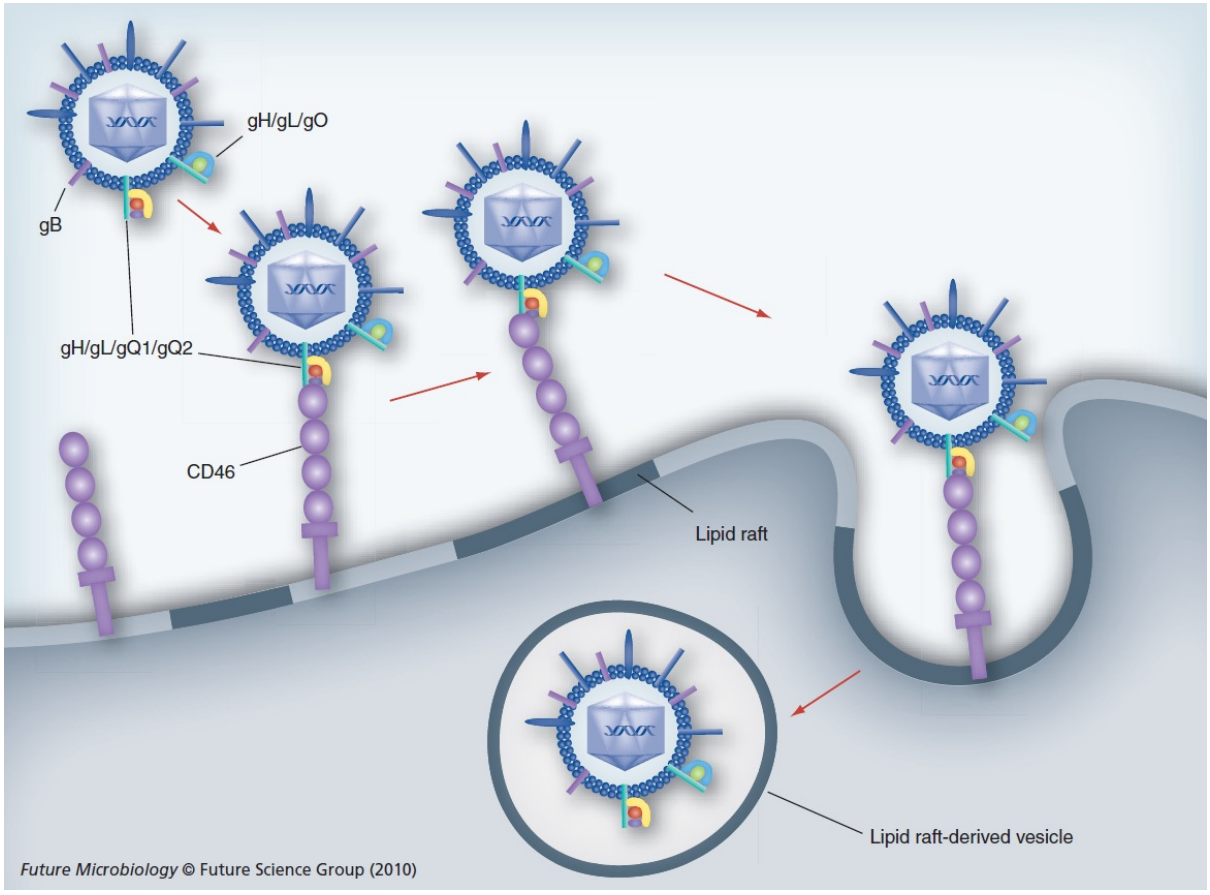


Figure 1.3 Human herpes virus 6 entry into host cells

This figure shows HHV6 enters the cell through interaction with CD46. Glycoprotein complex (the glycoproteins H, L and Q) of HHV6 acts as the viral ligand for human CD46. *(Tang and Mori, 2010)*

After binding to the cell membrane receptor, fusion of viral envelope is mediated through gH-gL-gQ (Figure 1.4). The nucleocapsid is transported via the cytoplasm to the nuclear pore complexes, followed by the release of the viral DNA genome into the nucleoplasm (De Bolle *et al.*, 2005b; Zhoua *et al.*, 2006). Within the nucleus, the virus exploits the host cellular transcription and translation apparatus for its gene expression in an orchestrated manner. Expression of immediate early (IE) gene (such as U90 transcription factor) is activated first (within a few hours after infection); which is followed by early (E) and late (L) gene expression. The IE proteins are trans activators of gene expression having a regulatory effect on expression of

other genes. The E proteins take part in viral DNA replication (via DNA polymerase); and the L proteins (structural protein) take part in the formation of the mature virus particles, such as U54 and U11 (tegument protein). While exiting the nucleus the mature capsids acquire a tegument and become encircled with a viral envelope containing glycoprotein spikes, which undergo sequential glycosylation in transport vesicles. The release of new virus progeny occurs through an exocytosis mechanism (Figure 1.4).

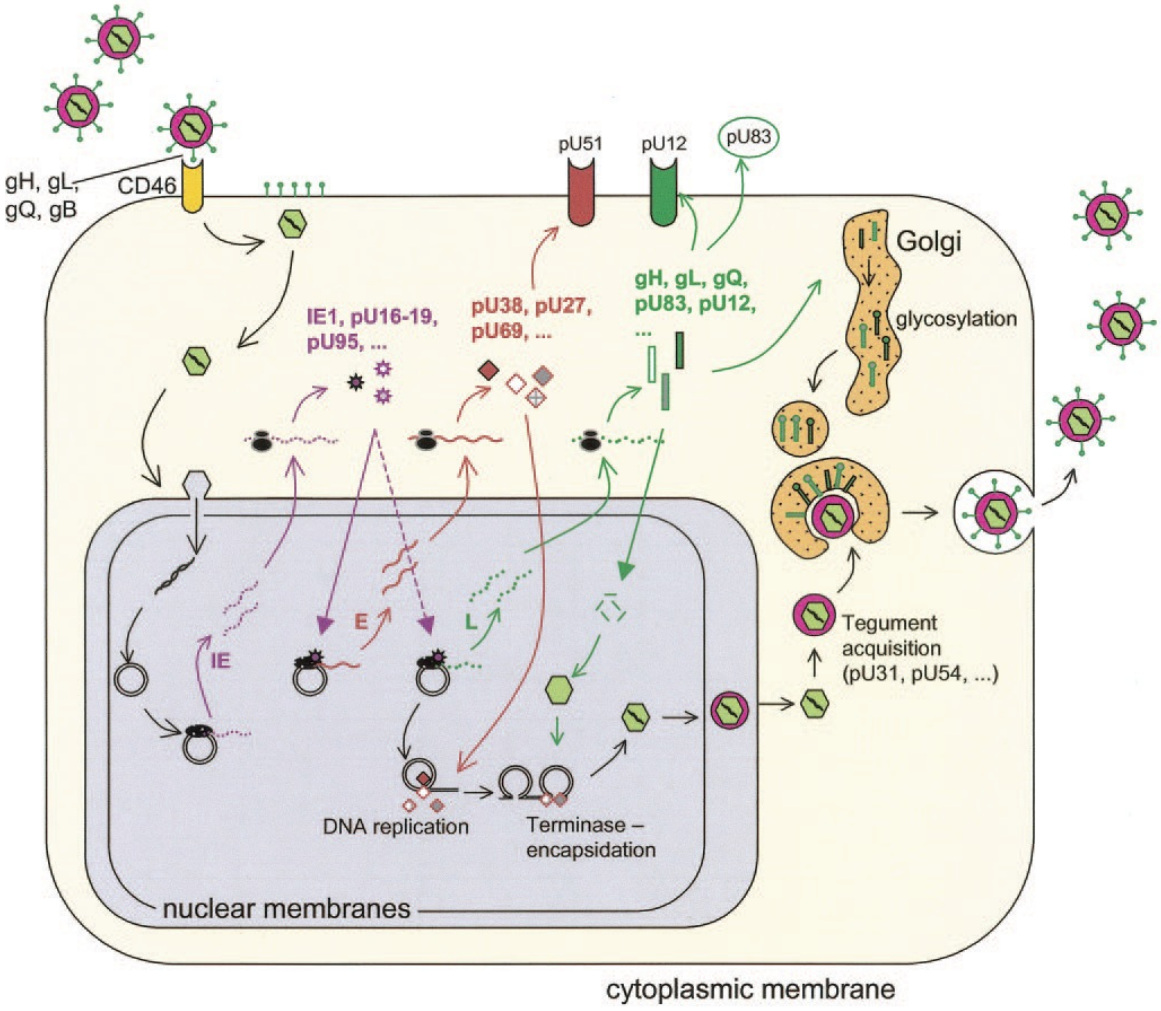


Figure 1.4 Representation life cycle of HHV6

Figure showing the schematic representation of the HHV6 lytic replication cycle. *(De Bolle et al., 2005)*

1.4 Epidemiology and Transmission of HHV6

HHV6 is a common virus, which is prevalent throughout the world. Seroprevalence is high (more than 90%) among the adult population, and seroconversion usually occurs before 2 years of age (Hall, 1997; Yoshikawa *et al.*, 1989). Maternal antibodies that are present in the new-born decline with advancing age and by six months may become undetectable. After that, children are at risk of primary infection. Pre-natal infection is also reported with detection of the virus in cervical swabs during pregnancy, suggesting the possibility of transplacental viral transmission (Okuno *et al.*, 1995; Caserta *et al.*, 2007). The commonest site of localisation of HHV6 is in salivary glands (Chen and Hudnall, 2006; Fujiwara *et al.*, 2000), therefore, the most likely source of person to person transmission is saliva. Unfortunately, there is no specific serological test to differentiate between the two HHV6 subsets. Generally, the acquisition of HHV6 occurs between 6 and 15 months of age. The incubation period before overt infection varies from 1-2 weeks (Enders *et al.*, 1990; Okuno *et al.*, 1989). Almost all roseola cases are caused by HHV6B infection (Braun *et al.*, 1997; Dewhurst *et al.*, 1993; Hall *et al.*, 1998). However, HHV6A is also found to be responsible for roseola in a few patients (Braun *et al.*, 1997; Dewhurst *et al.*, 1993). HHV6A sero-conversion is believed to happen following acquisition of HHV6B (De Bolle *et al.*, 2005). Reports suggest that HHV6A is more neurotropic than HHV6B (Hall *et al.*, 1998; Portolani *et al.*, 2001; Borghi *et al.*, 2005). Studies also showed a selective reactivation of HHV6A in immunocompetent patients while they are severely ill (Razonable *et al.*, 2002).

1.5 HHV6 Chromosomal Integration

Since the discovery of chromosomally integrated HHV6 (CIHHV6) in 1993, this uncommon phenomenon has raised the curiosity of researchers and troubled clinicians (Luppi *et al.*, 1993). Using FISH analysis, several studies have proposed that both HHV6A and B are capable of integrating into human chromosomes (Daibata *et al.*, 1998; Nacheva *et al.*, 2008; Torelli *et al.*, 1995) and transmitting vertically through the germ line (Tanaka Taya *et al.*, 2004). Previous studies also suggested the infrequent passage of viral genome through the germ line, resulting in a high prevalence of inherited-integrated HHV6. Reports from several British and US groups (Nam Leong *et al.*, 2007; Ward *et al.*, 2005) showed high HHV6 viral load (10^6-10^7 copies/ml) in peripheral blood of normal healthy donors (prevalence 0.8% - 1.5%), which is accredited to integrated-inherited HHV6 (Ward *et al.*, 2005). Interestingly, in hospitalised patients an even higher prevalence (2.9% - 3.3%) has been reported (Nam Leong *et al.*, 2007).

Chromosomal integration is one of the mechanisms by which HHV6 could accomplish its latency, mimicking inherited HHV6 (Clark *et al.*, 2006). Evidence that chromosomally integrated viruses are capable of producing free virus *in-vitro*, indicates that this could also happen *in-vivo* (Arbuckle *et al.*, 2010).

So far, CIHHV6 genotypes are not associated with any definite pathology. Most of the studies analysing the association of herpes viruses among unhealthy cohorts did not include detection of CIHHV6 as priority objective (Morissette and Flamand, 2010). The assumption that CIHHV6 DNA would certainly produce free virus leading to human infection followed by transcription of viral proteins and production of

cytokines leading to cell damage or triggering of an autoimmune response, is not well supported (Flamand *et al.*, 2010). A number of hypothetical explanations of the fate of CIHHV6 exists (schematically represented in Figure 1.5) (Morissette and Flamand, 2010). Activation of the telomeric regions at the sites of HHV6 insertion contributes to chromosomal integrity and cellular homeostasis, as well as encoding several transcripts (Calado and Young, 2009; Riethman, 2008). Others (Arbuckle *et al.*, 2010), suggest that since CIHHV6 is transplacentally transmitted, it may lead to immunological tolerance to several HHV6 antigens owing to the expression of the viral proteins during embryogenesis.

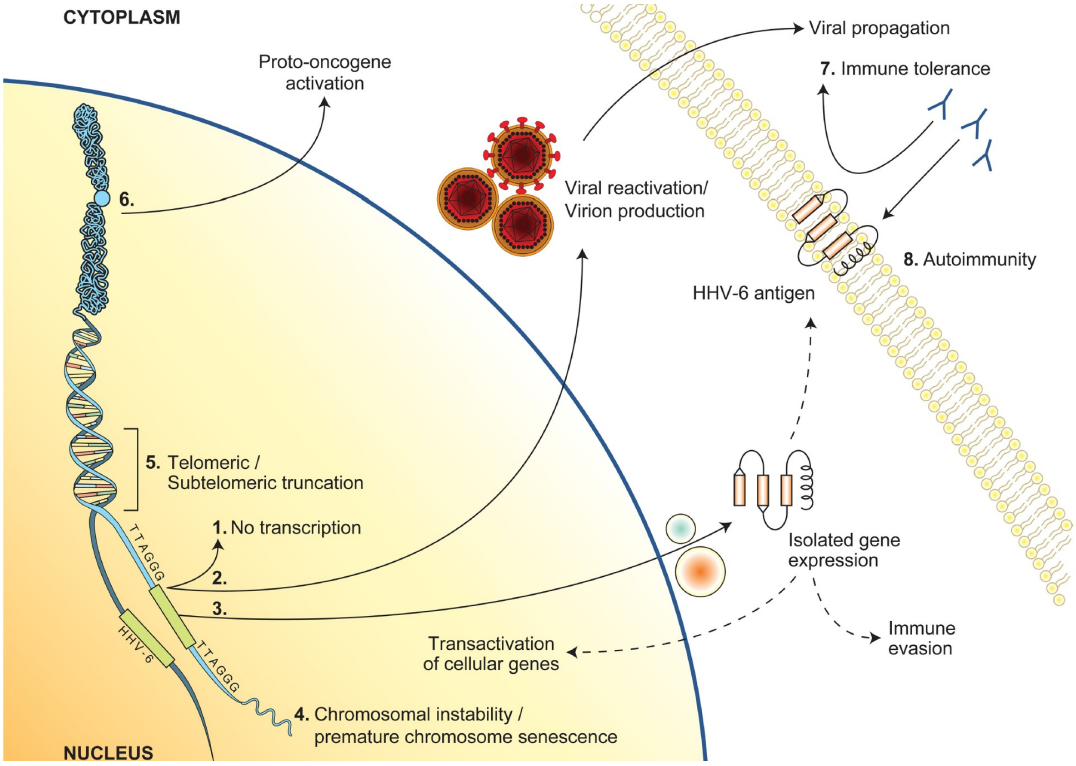


Figure 1.5 Schematic representation of the hypothetical cellular consequences of CIHHV6

(1) absence of viral gene transcription; (2) expression of gene, replication and production of virion; (3) expression of a subset of HHV6 genes; (4 and 5) the impact of integration of HHV6 on function and architecture of the telomere, and chromosomal stability; (6) activation of gene expression following integration in the cell; (7) development of immune tolerance following expression of HHV6 genes during embryogenesis; (8) damage of tissues or cells expressing HHV6 antigens by naturally developed immune response. *(Morissette and Flamand, 2010)*

1.6 Pathology and Treatment of HHV6

1.6.1 Pathology

Primary HHV6 infection in children is associated with fever and roseola (Figure 1.6; Yamanishi *et al.*, 1988). The typical manifestation of roseola is high temperature (≥ 40°C) for a few days; after that the fever subsides concurrent with appearance of a skin rash. The rash typically appears on the chest first, and after that extends to the face, legs and arms. It contains small sores that do not itch and more often vanishes within a few days to a week. Some cases of primary HHV6 infections manifest with variable clinical pictures; such as, absence of skin rash and immediate onset of high pyrexia. The prognosis of roseola is generally good, but may become worse with a number of complications. The commonest complication results from CNS infection, manifested by seizures and others signs of encephalitis (Asano *et al.*, 1994; Hall *et al.*, 1994; Suga *et al.*, 1993; Ishiguro *et al.*, 1990). Primary HHV6 infection may be associated with a mild hepatic dysfunction, which may proceed to a severe form of fulminant hepatitis (Ohashi *et al.*, 2004; Ishikawa *et al.*, 2002; Mendel *et al.*, 1995; Sobue *et al.*, 1991; Asano *et al.*, 1990).

Briefly, HHV6 infection can cause a self-limiting rash-like illness in infancy but is followed by lifelong asymptomatic infection, typical of other herpes viruses. HHV6 reactivation from latency can lead to severe diseases in immunocompromised individuals, such as encephalitis (Zerr *et al.*, 2001; Singh and Paterson, 2000; Corti *et al.*, 2011), hepatitis (Härmä *et al.*, 2006; Chang *et al.*, 2009) and pneumonitis (Cone *et al.*, 1993; Carrigan *et al.*, 1991) in AIDS patients and transplant recipients (Figure 1.6).

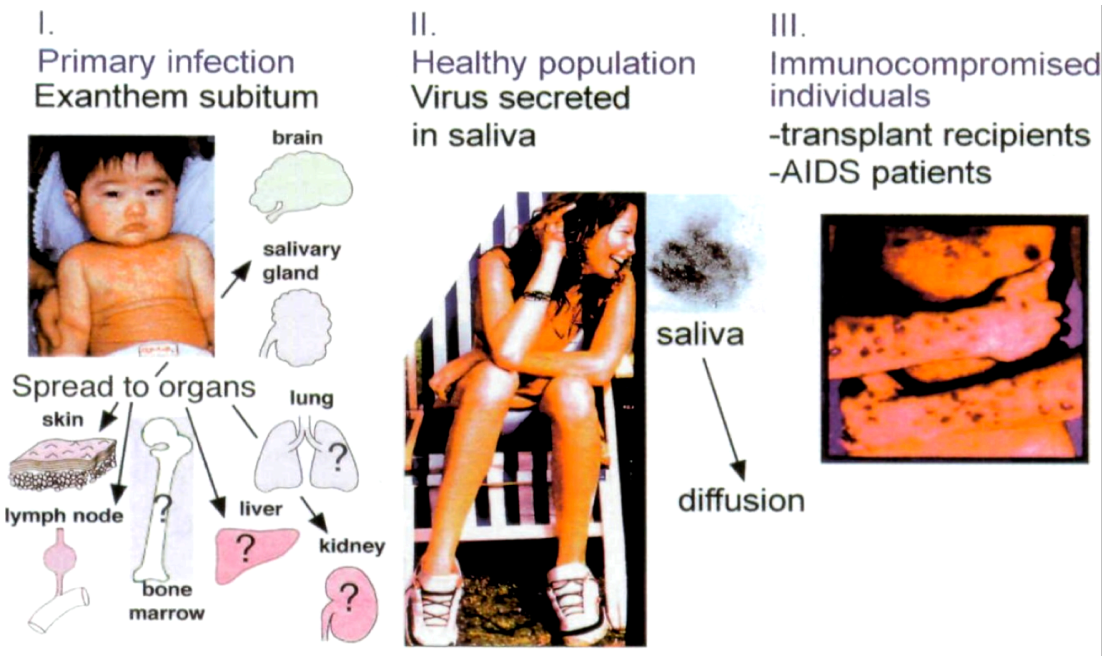


Figure 1.6 Stages of HHV6 Infection

Figure showing stages of the natural course of HHV6 infection: I. Primary HHV6 infection occurs in new-borns, commonly causing roseola (exanthem subitum), and may spread to organs. II. In healthy adults, HHV6 may persist in the lymphatic system and salivary glands; the commonest source of transmission is via saliva. III. HHV6 infection or reactivation occurs in immunocompromised patients either following transplantation (therapeutic) or HIV infection. *(Campadelli-Fiume et al., 1999)*

1.6.2 Treatment

In immunocompetent individuals, HHV6 infections may resolve without any treatment in a self-limiting manner. However, in immunocompromised individuals reactivation of HHV6 infection from latency can lead to severe disease. Until now, no specific medication is available for treating HHV6 infection after transplantation. Nevertheless, *in-vitro* experiments have revealed a number of drugs (ganciclovir,

cidofovir and foscarnet) are active against the virus (De Clercq *et al.*, 2001). Antiviral therapy in post-transplant HHV6 infection is very limited in clinical practice. There are reports of successful application of ganciclovir and foscarnet in symptomatic HHV6 infection among recipients of bone marrow transplantation (Bethge *et al.*, 1999; Zerr *et al.*, 2002). There are also reports of decreased HHV6 viral loads following ganciclovir therapy among liver transplant patients (Mendez *et al.*, 2001). So far, no information is available regarding the treatment or prevention of HHV6 in transplant patients in controlled studies.

In addition to the cost of antiviral drugs, there are many side effects as well as their lack of effectiveness for all viruses. Therefore it is important to understand the immune response to HHV6, since this is the natural way to control this virus without incurring side effects.

1.7 Adoptive immunotherapy

In immunocompromised individuals (such as transplant recipients or HIV patients), viral reactivation of EBV and HCMV is associated with high morbidity and even mortality due to lack of cellular immunity against viral antigens. Because antiviral drugs are not effective enough to eliminate the virus besides being costly and having many side effects, an alternative had to be found. Adoptive T cell immunotherapy is a promising therapeutic approach for the treatment of diseases associated with reactivation of herpes viruses in immunosuppressed patients. The purpose of adoptive immunotherapy is to passively immunise an individual with an external source of specific T cells that could be used as an anti-viral treatment and be capable

of providing protection. This can be done by isolation of T cells from a healthy donor previously exposed to the virus, followed by *in-vitro* stimulation of these cells with specific viral peptides. After that, specific polyclonal T cells are expanded with addition of cytokines (such as IL-2) to help T cell growth and differentiation. Finally, these T cells can be re-injected into the same recipient directly after transplantation (Figure 1.7) (Leen et al., 2007). Adoptive immunotherapy is well characterised against EBV and HCMV with encouraging results (Leen et al., 2009; Nickel et al., 2009). The first promising results using antigen-specific adoptive immunotherapy in humans were observed in the early 1990s using passively transferred, *in-vitro* expanded donor PBMC. HCMV-specific cytotoxic T lymphocytes for the treatment of HCMV infection in immunocompromised allogeneic BMT recipients have been shown to provide immunity to transplant recipients (Greenberg *et al.*, 1991; Riddell *et al.*, 1991; Riddell *et al.*, 1992).

At present, adoptive T cell immunotherapy can successfully prevent and treat fatal infections in the immunocompromised host. These benefits have been restricted to EBV, HCMV and adenovirus (Leen *et al.*, 2006; Karlsson *et al.*, 2007; Leen *et al.*, 2009; Barker *et al.*, 2010). In the case of HCMV, the two major protein of the CD8+ T cell immune response, IE1 (UL123) and pp65 (UL83), have been used successfully in adoptive transfer (Bao et al., 2008). This therapy has not been available for HHV6 infections; in part due to lack of identified immunodominant CD8+ T cell epitopes with defined protective activity. Adoptive immunotherapy can be extended to HHV6 to develop BMT engraftment and control recipients' viral reactivation.

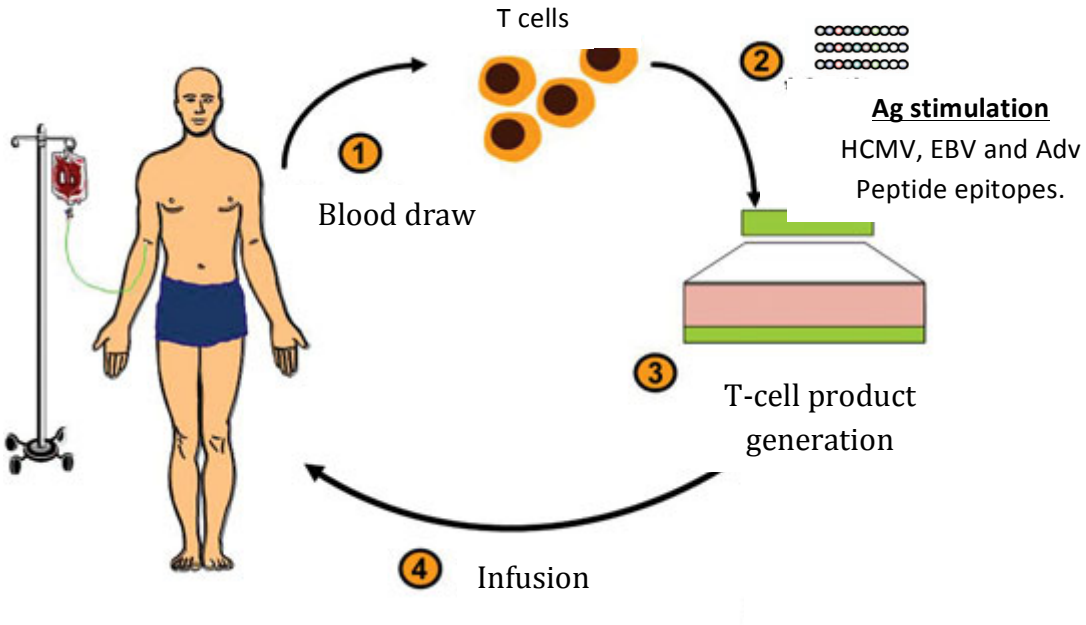


Figure 1.7 Illustration diagram of the general steps in an adoptive immunotherapy.

Figure showing: 1. T cell isolation from the healthy donor; 2. Stimulation of T cells with viral antigens; 3. Expansion of polyclonal T cells; 4. Re-infusion of T lymphocytes to the recipient after transplantation. (*Adapted from Reddy et al., 2001)*

1.8 Immunity against herpes viruses

The immune response against herpes viruses involves all the arms of the immune system including both innate and adaptive immune responses. However, antigen specific CD8+ T cells, CD4+ T cells, and Natural Killer (NK) cells are the three main immune cells that play an important role in the immune response to control herpes virus infection; humoral immunity is also involved in providing such protection.

1.8.1 Innate immunity

The innate immune systems provide a first line of defence against infection. Unlike the adaptive immune system, which provides long-lasting or protective immunity to the host, the cells of the innate system recognise and respond to pathogens in a generic way. Herpes viruses induce an innate immune response that will control viral replication before the establishment of the adaptive immune response. Innate immune cells do not recognise every antigen that is expressed on microorganisms but instead recognise some microbial molecular signatures that are shared by large groups of microorganisms through pattern-recognition receptors (PRRs).

Recognition of herpes viruses by Pattern-recognition receptors

PRRs are expressed on most innate immune cells such as macrophages and dendritic cells and are expressed constitutively. The innate immune system is activated when the infection is sensed by PRRs, via detection of specific components of the virion known as the pathogen-associated molecular patterns (PAMPs). Many PRRs are involved in recognition of herpes viruses infection, however Toll-like receptors (TLR) are the most studied PRRs (Takeuchi and Akira, 2010). Genomic DNA of herpes virus can be recognised by endosomal TLR, such as TLR9 (Tabeta *et al.*, 2004). RNA produced during herpes virus replication could also be recognised by other PRRs such as retinoic acid-inducible gene I (RIG-I) and RIG-I like receptors (RLRs) (Paludan *et al.*, 2011). Following the binding of the ligand to its receptor, an intracellular signal will be released to enable the expression and secretion of pro-inflammatory cytokines such as type I interferon (type I IFN) that includes (IFN-α and IFN-β).

Recognition of HHV6 by the Pattern-recognition receptors

Expression of TLRs for the reconnaissance of PAMPs and induction of an antimicrobial interferon response were investigated in CD4+ T cells and dendritic cells. In CD4+ T cells infected with HHV6A, it was demonstrated that TLR-9 was over-expressed and induced apoptotic signal transduction (Chi *et al.*, 2012). TLR signalling leads to the expression of antiviral defences via type I IFN that inhibit viral replication. It has been shown that HHV6A and HHV6B were able to limit this by acting on the response signal or the effect of these cytokines (Murakami *et al.*, 2010).

Role of NK cells in immunity to herpes viruses

NK cells are a part of the innate immune system and provide a protection against viral infection without previous stimulation. NK cells play a key role in early herpes virus infection control. For example, observations from the murine model of HCMV (mCMV), provide direct evidence of the importance of NK cells in cytomegalovirus immunity; it has been used to study the role of NK cells in CMV immune surveillance. Mice were infected with mCMV and, after it became latent, T cells and NK cells were depleted. A high viral replication was found just three days after depletion (Tay and Welsh, 1997). In NK cell deficient patients, studies have shown that severe herpes infections can occur (Notarangelo and Mazzolari, 2006).

Role of NK cells in immunity to HHV6

NK cell deficient patients are more susceptible to herpes virus diseases, including HHV6 (Orange, 2012). NK cells respond rapidly following infection and their activity is greatly increased by pro-inflammatory cytokines. The NK response reaches a peak

in HHV6 infected children during the acute phase and does not decline until the adaptive immune response takes over (Takahashi *et al.*, 1992; Kumagai *et al.*, 2006). These findings suggest that NK cells are one of the elements of the control of proliferation of HHV6 in a primary infection (Kumagai *et al.*, 2006). HHV6 infection also enhances NK cell activity, possibly by inducing IL-15 production, which stimulates the proliferation of NK cells and T cells (Flamand *et al.*, 1996; Atedzoe *et al.*, 1997). IL-15 also stimulates the production of a Type II interferon (IFN-II) that include IFN-γ (Gosselin *et al.*, 1999). IFN-I and IFN-γ promote the expansion and activation of CD4+ and CD8+. Little is still known about the NK cell mechanisms that eliminate HHV6 infected cells. It has been suggested that NK cells are very effective in killing HHV6 infected cells, and possibly play a main role in controlling viral infection (Malnati *et al.*, 1993).

1.8.2 The adaptive immune response to herpes viruses

The major functions of the adaptive immune system are to generate an immune response that is capable of eliminating specific pathogens or pathogen infected cells and to develop memory cells.

Effector cells

T and B cells are the major lymphocyte types that have a vital function in the adaptive immune response. T cells are involved in cell-mediated immunity, while B cells in the humoral immunity. T and B cells are created via stem cells in the bone marrow. T lymphocytes travel to thymus and develop in there. T and B cells are located in lymphoid tissues in at least three differentiation stages; initially, a naive

cell which has not activated yet with an antigen. Then, effector cells which have been activated and involved in pathogen removing. Finally, memory cells which are the cells that met specific antigen during a previous infection (Janeway *et al.,* 2001).

Antigen processing and presentation

The MHC system plays an important role in the immune responses by recognising non-self antigen, which is expressed on hosts' cells. The MHC molecules can be split into two classes; MHC class I and MHC class II. CD8+ and CD4+ T cells have been shown to be important against herpes virus replication. CD8+ T cells recognise short viral peptides presented by class I MHC molecules, whereas CD4 T cells recognise longer peptides presented by class II MHC molecules (Figure 1.8). Herpes virus-specific CD4+ T helper cells prevent herpes virus infection through secreting tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and other cytokines. However, some herpes viruses like HCMV have the ability to escape from the immune system via decreasing MHC Class I and MHC Class II expression on infected cells, which results in a decrease in cytotoxic CD8+ T cell surveillance; CD8+ T cells are the primary cells that recognise herpes virus infected cells (Hertel *et al.*, 2003).

MHC class I

MHC class I molecules are expressed by most nucleated cells. MHC class I bind peptides from intracellular or endogenously synthesised antigens. The host cells breakdown the non-self protein, for example virus protein, to a small pieces (8-13 amino acids in length) called peptides. Peptides have anchor residues at 2nd and C-terminal positions. These peptides are generally made by the proteasome then

released into the cytosol. Viruses are unable to make own proteins so use host cell ribosomes. Endogenously synthesised proteins labelled for degradation by ubiquitin. Also, mis-folded or mis-translated proteins degraded prematurely. Proteins are broken by the proteasome (via proteolysis): a multi-subunit enzyme complex. Selected peptides are transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER), where they are loaded onto MHC class I molecule. The MHC class I-peptide complexes leaves the ER and transported to the Golgi apparatus and then to the surface of the cell. Complex of MHC class I-foreign peptide then will be screened by CD8+ T cells for foreign peptides (Klein *et al.*, 2000).

MHC class II

MHC class II are expressed on professional APCs of the immune system that take up exogenous or extracellular antigens; dendritic cells, B cells, macrophages and activated T cells. The exogenous antigens that are taken up by APCs are sequestered into endosomes. Class II MHC molecule is synthesised in the ER and then travel via the Golgi apparatus. Newly synthesised class II in ER prevented from binding endogenous peptides by the invariant chain (Ii). Ii is digested by acid proteases in the endosomal compartment. Leaves CLIP (Class II associated invariant-chain peptide) in peptide binding groove. HLA-DM molecule that is synthesised in the ER helps dissociate CLIP, freeing the binding groove to bind exogenous peptide. Traffic to endosomal compartment where acid pH, proteases release invariant chain and permit binding of exogenous peptide. MHC peptide complexe are then traffic to cell surface and presented to CD4+ T cells (Klein *et al.*, 2000).

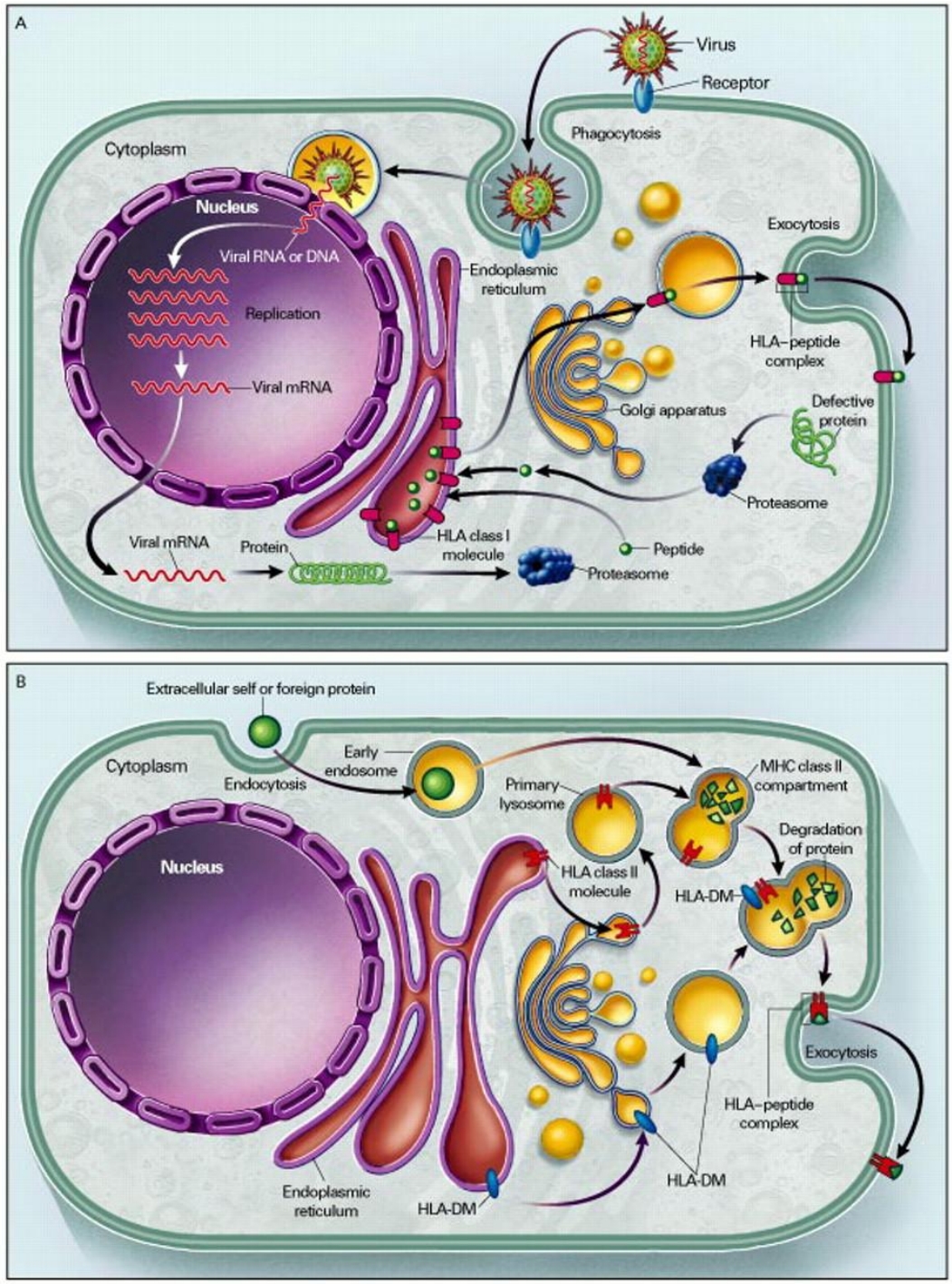


Figure 1.8 MHC Class I and MHC Class II Antigen Processing Pathways

Figure showing antigen presentation to the immune system: **(A)** The MHC I pathway presents intracellular or endogenously produced proteins that split into peptides (8-13 aa) and then absorbed into the endoplasmic reticulum (ER) through transport-associated protein (TAP) molecules; MHC I pathway leads to the cell-mediated/cytotoxic CD8 T cell mediated immune response. **(B)** The MHC II pathway presents extracellular or exogenous antigens in antigen presenting cells then presenting them to CD4+ helper T cells, leading to an antibody biased immune response. *(Klein et al., 2000)*

T lymphocytes

T lymphocyte cells have different types, two of which are CD4 that recognise foreign antigen in association with class II MHC and CD8 that recognise antigen in association with class I MHC. T are expressed a specific receptor called TCR (T Cell Antigen Receptor). TCR has alpha and beta polypeptide chains that have variable domains. The variable domain has 3 hyper-variable regions which can recognise specific antigens. After antigen recognition, CD3 molecule pass signal into the T lymphocyte that led to T lymphocytes activation, proliferation, differentiation or cytokine production (Okkenhaug *et al.*, 2004).

Cytotoxic CD8 T lymphocytes can attack cells that carrying foreign molecule and cause rapid death to these abnormal cells, which eliminate pathogen in a short time (Janeway *et al.*, 2001). CD4 lymphocyte called T helper cell because is not normally cytotoxic but help other cells, such as macrophage and CD8 T cells, to induce the killing activity and eliminate the pathogen. CD4 T helper lymphocytes have two types, which are Th1 and Th2. Th1 is effective to intracellular pathogen, while Th2 response is more effective to extracellular pathogens. Regulatory T cells (Treg) are a third type of T lymphocyte that can suppress the immune responses, and control the autoimmune disease development (Janeway *et al.*, 2001).

Role of CD8+ T cells in herpes virus infection and HCMV

CD8+ T cells are known to be involved in herpes virus immunity. This evidence, for example in HCMV, comes from both transplant patients (Reusser *et al.*, 1991) and mCMV, the animal model for HCMV infection. In HCMV seropositive autologous

stem-cell transplant recipients, HCMV specific T cell responses were sufficient for controlling HCMV infection (Reusser *et al.*, 1997; Rauser *et al.*, 2004). There is, however, a study by Boeckh (2003) that argues that, after allogeneic stem cell transplantation, recipients who lack HCMV specific CD4+ and CD8+ T cells are at a greater risk of developing infection (Boeckh *et al.*, 2003). In the animal model, though, there is more direct evidence because it is possible to deplete T cells and infect mice with mCMV. CD8+ T cells protect a mCMV infected mouse from disease, as it has been shown that depletion of CD8+ T cells is associated with a remarkable increase in morbidity after mCMV infection. Therefore, CD8+ T cells are important in immunity against mCMV (Reddehase *et al.*, 1985). This evidence led to the development of protocols for reconstituting HCMV-specific CTL immunity in transplant recipients by means of adoptive transfer of cloned CTL against the HCMV structural protein pp65 (Wills *et al.*, 1996b).

Research on the specificity, phenotype and function of HCMV specific CD8+ T cells have been going on for over three decades. The immediate early protein (IE) (Borysiewicz *et al.*, 1988) and the tegument protein pp65 (UL83) (McLaughlin-Taylor *et al.*, 1994) were among the first viral proteins recognised as targets of the CD8+ T cell response to HCMV. Then, many studies have concluded that T cell reactivity against pp65, pp50 and IE1 appears to constitute an important part of CMV-specific cytotoxic T lymphocyte (CTL) responses in the healthy population carrying the virus (Elkington *et al.*, 2003; Kern *et al.*, 1999; Khan *et al.*, 2002a; Khan *et al.*, 2004). There is conflicting evidence regarding the role of pp65 responses as marker of clinical importance. Some research has suggested the importance of pp65-specific CTL

responses (Riddell *et al.*, 1992; Lacey *et al.*, 2006); other studies have shown that IE1-specific CTL responses are important, while pp65-specific CTLs are not (Bunde *et al.*, 2005). Apparently, pp65-specific and IE1-specific T cell responses are of advantage throughout different phases of viral activity; perhaps the former is important in identifying freshly infected cells, as virion-derived pp65 is readily available for processing before IE1 can be expressed. While virus reactivates from latency, IE1 responses could be important, as the processing and presentation of IE1 antigen would be expected to occur sooner than that of pp65. In fact, IE1-specific responses would likely increase progressively over time after primary HCMV infection, while pp65 responses would peak near the beginning and gradually diminish thereafter (Khan *et al.*, 2007). It also has been reported that successful CMV-specific immune responses in healthy carriers would depend on a strong CTL response to a broad antigenic repertoire (Elkington *et al.*, 2003).

Role of CD4+ T cells in herpes virus infection and HCMV

Experiments with an animal model have also been done for HCMV-specific CD4 T helper cells, the result being that depletion, and/or dysfunction, of CD4+ T cells and antibodies can result in HCMV-associated disease (Waisman *et al.*, 2008). The triggering of killing potential of these CD4+ T cells can be via direct recognition of endogenous antigen in addition to exogenous antigens, as shown by studies of the HCMV Glycoprotein B (gB), which is processed and expressed efficiently by MHC class II of infected cells (Hegde *et al.*, 2005). Although the response of CD4+ T cells can be demonstrated against a wide range of HCMV antigens, the main responses

are found to be against pp65 and gB, which are also strong antigens for CD8+ T cells. IE1, though a strong antigen for CD8+ T cells, is less stimulatory than pp65 and gB for CD4+ T cells (Sylwester *et al.*, 2005). This may be because IE-1 does not access the class II processing pathway as readily as pp65 and gB, which are both structural components of the virus particle and more classical exogenous antigens. Recent studies on normal asymptomatic seropositive individuals and women throughout pregnancy showed that CD4+ cytotoxic T lymphocyte responses were mainly directed towards the gB antigen (Hopkins *et al.*, 1996).

1.8.3 The adaptive immune response to HHV6

However, mutual development of adaptive immune responses by the host, as reviewed for HCMV (Basta and Bennink, 2003), has not been investigated for HHV6. Regarding immune modulation, HHV6 can infect a number of immune cells implicated in the production of both cellular and humoral adaptive immune responses. The adaptive immune response against herpes viruses is very important in the control of viral replication in primary infections or during reactivations. The characterisation of these responses in EBV and HCMV allowed the development of immunotherapies (Leen *et al.*, 2007; Leen *et al.*, 2009). The specific adaptive immune response to HHV6 is very little known. The characterisation of the response to HHV6 is complicated due to its tropism for CD4+ T cells (Takahashi *et al.*, 1989) and cytopathic effects following infection (Flamand *et al.*, 1995; Gupta *et al.*, 2009; Li *et al.*, 2011). In addition, owing to the fact that almost all of the population is infected with HHV6 (De Bolle *et al.*, 2005), access to non-infected individuals who

could provide information on the basal level of immune responses remains very difficult. Although the narrow host range of HHV6 has limited the utility of animal models, severe combined immunodeficiency (SCID) mice implanted with human fetal thymus and liver (huSCID Thy/Liv mice) have provided a first useful model of HHV6 infection (Gobbi *et al.*, 1999). In this model, HHV6 subgroup 6A or 6B, efficiently infected the human thymic tissue implanted in SCID-hu Thy/Liv mice, leading to thymocyte depletion and reduced thymic output. However, data on T cell responses are limited regarding HHV6 because it is restricted to primates (Lusso *et al.*, 1990). Therefore, there is so far no good animal model for infection and although the virus is able to penetrate rodent cells, it is not capable of replication (Santoro *et al.*, 1999; Mock *et al.*, 2006). The primary target cells for both HHV6A and 6B are CD4+ cells (Lusso *et al.*, 1988; Takahashi *et al.*, 1989), although the A strains are capable of broader tropism, including natural-killer cells, γδ T cells and CD8+ cells (Lusso *et al.*, 1991; Lusso *et al.*, 1993; Lusso *et al.*, 1995). HHV6 infection enhances production of inflammatory cytokines and chemokines, including IFN-α, interleukin [IL]-1β, IL-6, IL-15, IL-21 and RANTES (Wang and Pellett, 2007). Among them, regulated upon activation, a normal T cell expressed and secreted (RANTES), a proinflammatory β-chemokine, is known for inducing local responses by selectively attracting monocytes (the target for HHV6 latency) and lymphocytes (a vehicle for virus spread; Figure 1.9). Interleukin-1β (IL-1β) enhances inflammatory and interferon-γ (IFN-γ) responses, and is needed for IL-6-dependent, B-lymphocyte immune responses. Both IFN-α and IFN-β inhibit HHV6 replication *in-vitro* (Takahashi *et al.*, 1992). However, tumour necrosis factor-α (TNF-α), normally an antiviral cytokine, may enhance release of extracellular HHV6 by stimulating monocyte differentiation

(Arena *et al.*, 1997). HCMV tegument and nucleo-capsid proteins are better than the glycoproteins at inducing T cells proliferation (Ljungman *et al.*, 1985). Likewise, neither HHV6A nor HHV6B glycoproteins induce T-lymphocyte proliferation in healthy adults; as an alternative, these antigens inhibit T-lymphocyte proliferation to antigens or mitogens (Horvat *et al.*, 1993). Antigen preparations mainly composed of the tegument and nucleocapsid proteins of HHV6A and HHV6B induce T-cell proliferation in healthy seropositive adults and children (Yakushijin *et al.*, 1991; Soldan *et al.*, 2000). Studies were conducted in children infected by HHV6. Specific T cell response to HHV6B is established in most children within two weeks after the first symptoms (fever) (Kumagai *et al.*, 2006). However, this anti-HHV6 response appears to occur late compared to other human herpes viruses. The reasons for this delay are not clear, but do not appear to be due to a dysfunction of T cells induced by HHV6. In general, the memory immune responses seen in children seem to be greatest between the ages of 3 and 12 (Koide *et al.*, 1998). Analyses by flow cytometry (FACS) on PBMC showed similar frequencies of CD4 + T cells and CD8+ cells specific for HHV6 in healthy adults and children post-infection (Koide *et al.*, 1998; Haveman *et al.*, 2010). Both MHC dependent and independent cytotoxicity have been observed for HHV6-specific CD4+ T cell clones, suggesting heterogeneous functions of CD4+ T cells (Yakushijin *et al.*, 1992). However, some T cell clones with activity to HHV6 also responded to HCMV and HHV-7, which might indicate some cross-reactivity (Yasukawa *et al.*, 1993).

Modulation of the host immune response represents an important mechanism of evading the immune response or to make a good environment where the viruses can

survive, grow and persist (Vossen *et al.*, 2002), such as herpes viruses that persist for the lifetime of the host (Hengel *et al.*, 1998). HHV6 has been characterised as having immune modulatory properties mediated by direct effects in infected cells or indirectly on uninfected cells. One of the most intriguing features of HHV6 is its ability to evade host immune defences. HHV6 induces CD4+ T-lymphocyte depletion. Interestingly, HHV6A and 6B infection diverts Class I MHC molecules from the cell surface into a lysosomal compartment via U21 that binds and down regulates MHC class I, which possibly aids the viruses to escape immune detection (Glosson and Hudson, 2007). As mentioned above, the HHV6 receptor is CD46, a molecule that is critically important in the immunological synapse, IL-2 and IFN-γ production. However, HHV6 infection results in a dramatic down modulation of CD46 expression (Santoro *et al.*, 1999). Another intriguing CD46-mediated mechanism that might operate is induction of a T-regulatory cell 1 phenotype (Tr1) (Kemper *et al.*, 2003). Active suppression by (Tr1) cells has emerged as an essential factor in the control of auto reactive cells (Shimon, 2000). From studies of T-cell differentiation via specific monoclonal antibodies Kemper illustrated that co-engagement of CD3 and CD46 (the complement regulator) in the presence of IL-2 induces a Tr1-specific cytokine phenotype in CD4+ T cells. Stimulation of CD3/CD46 induced IL-10-producing CD4+ T cells to proliferate strongly, suppress activation of bystander T cells and acquire a memory phenotype (Kemper *et al.*, 2003). Wang *et al* have reported the induction of IL-10 secreting CD4+ T cells by HHV6 infection (Wang *et al.*, 2006).

HHV6 reactivation from latency can lead to severe disease in immunocompromised persons, such as T-cell deficient transplant recipients. This suggests that host immunity plays a key role in maintaining the normal disease-free host-virus balance. Unlike for most other HHVs, there is very little information on T cell antigens and epitopes encoded by HHV6; this will be addressed in this study.

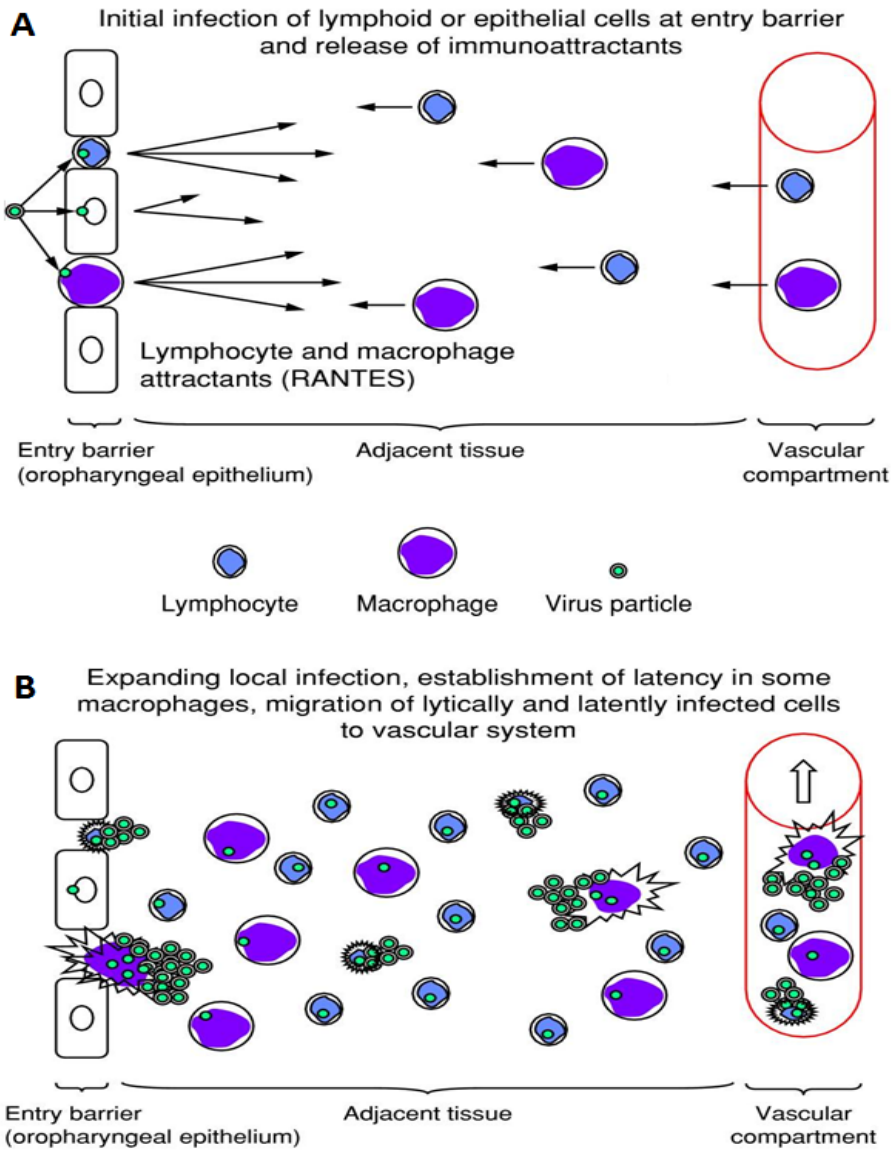


Figure 1.9 Immunobiological events of HHV6

This figure shows Immunobiological events during early primary HHV6 infection (A), and establishment of latency (B). *(Wang and Pellett, 2007)*

1.9 Aims and objectives

At the start of this project very little was known about the T cell response to HHV6. The immunogenic antigens had not been identified, and there was no information on potential antigenic peptides, HLA restricting alleles and strength of responses. However, based on the well-characterised cellular immune response to other human herpes viruses, particularly HCMV and EBV, it was hypothesised that HHV6 will be immunogenic for T cells. The aim of this thesis was to begin to characterise the T cell response to HHV6. There were three main approaches:

1. To investigate if T cell responses to HHV6 could be detected directly *ex-vivo* in healthy donors.
2. To *In-vitro* expand and analyse T cell responses to HHV6B antigens U11, U39, U54 and U90 in a panel of healthy donors.
3. To Identify and Characterise CD8+ T cell responses to HHV6B antigens U11 and U90.

It was hoped that the data generated in this thesis would provide new information and contribute to the existing information on the interaction between herpes viruses/HHV6 and the immune system as well as the potential to use this information to develop adoptive T cell therapy for diseases linked to HHV6 proliferation.

Chapter 2

2. Materials and Methods

2.1 Blood donors

Healthy adult volunteers (between 20 to 60 years old) were recruited for this study. All blood samples were obtained after formal consent was taken from each participating donor according to the Helsinki Declaration. The study was approved by the Liverpool Adult Research Ethics Committee (RCE reference 2K/175). All donors were HLA typed using a PCR-based protocol by the NHS Tissue Typing laboratory based in the Clinical Immunology Department, Royal Liverpool and Broadgreen University Hospital Trust.

2.2 Peripheral blood mononuclear cells preparation

Human peripheral blood samples were obtained from healthy donors, ranging in volume from 10 - 50 ml. Blood was taken by venipuncture into preservative free heparin, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation using Ficoll-Paque^{TM} Plus (GE Healthcare Biosciences AB, UK). Blood was gently layered onto an equal volume of Ficoll-Paque^{TM} Plus and centrifuged at 500 x g for 20 minutes at room temperature (RT), with the brake off. PBMC were harvested from the interface and washed twice with 20 ml RPMI 1640 media (Sigma, UK), followed by centrifugation at 500 x g for 10 minutes with brake on. Cell pellets were then re-suspended in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum [FCS], 2 mM Glutamine, 50 IU/ml Penicillin and 50 µg/ml Streptomycin (R10 growth medium). The yield of PBMC was determined using a haemocytometer. PBMC were then either used

immediately or cryo-preserved in liquid nitrogen in freezing media (R10 containing 10% DMSO and a further 10% FCS). Cryo-preserved PBMC were thawed and washed, prior to use.

2.3 Peptides

Single tube peptide mixes (pepmixes), containing 15-mer peptides overlapping by 11 amino acids (aa) spanning the whole protein were obtained from JPT Technologies (JPT Peptide Technologies GmbH, Berlin, Germany) for the following viral antigens:

HHV6B:	U11, U39, U54 and U90
HCMV:	IE1, IE2, pp65 and gB
Polyoma BK virus:	VP1

JPT pepmixes were dissolved in dimethylsulphoxide (DMSO) at a concentration of 5 mg/ml and stored at -20°C. Individual 15-mer peptides, for HHV6B antigens U11 and U90 (concentration of 10 mg/ml), again overlapping by 11 aa, were provided by Dr Ann Leen (Baylor College of Medicine, Houston, USA). These individual U11 and U90 15-mer peptides were used to generate mini-pools of 16 peptides, such that there were 30 mini-pools for U11 and 33 for U90, with each 15-mer represented in 2 mini-pools. Peptide pools were prepared in RPMI 1640 medium, with each peptide at a final working concentration of 10 µg/ml. Selected individual 9-mer peptides for HHV6B U11 and U90 were purchased from Alta Bioscience Ltd (Birmingham, UK) and reconstituted in DMSO to a final concentration of 5 mg/ml.

2.4 Cell lines

Phytohaemagglutinin (PHA) blasts were generated from donor PBMC. $2x10^6$ PBMC in 2 ml T cell media (RPMI 1640 medium supplemented with 5% heat inactivated Human AB Serum, 2 mM Glutamine, 50 IU/ml Penicillin and 50 μg/ml Streptomycin were treated with 5 μg/ml PHA and maintained at 37°C in a 5% CO_2. T cell media was replenished every 3 days. PHA blasts were used in T cell assays from day 3.

2.5 Virus stocks

HHV6B virus strain Z29 was generated as culture supernatant from HHV6B Z29 infected SupT1 cells (Mayne *et al.*, 2001), obtained from the HHV-6 Foundation (Santa Barbara, CA 93101 USA). Briefly, HHV6B-Z29 infected SupT1 cells were maintained for 6 days in R10 growth media. Virus-containing supernatant was then harvested, centrifuged at 400 x g for 5 minutes to remove cells, before being filtered through a 0.45 μm Minisart® NML Syringe Filter (Sartorius UK Ltd, UK). Virus containing supernatant was stored in single use aliquots at -80°C.

Virus containing supernatant from HHV6B strain HST infected cells was provided by Dr Andreas Moosmann, (Helmholtz Zentrum München, Munich, Germany).

2.6 Flow Cytometry

Flow cytometric analysis in this project was carried out on BD FACS Calibur (BD Biosciences). Different subsets of lymphocytes were defined using Forward Scatter (FSC) and Side Scatter (SSC) based gating. Fluorescence emissions of different fluorochromes were measured at different wavelengths as follows: fluorescein

isothiocyanate (FITC) and Alexa fluor 488 in FL1 at 519nm, phycoerythrin (PE) in FL2 at 578nm, peridinin chlorophyll protein–cyanine 5.5. (PerCPCy5.5), phycoerythrin with cyanin-5 (PECy5) and phycoerythrin with cyanin-7 (PECy7) in FL3 at 695nm, and allophycocyanin (APC) and Alexa fluor 660 in FL4 at 660nm (Baumgarth and Roederer, 2000). Unstained cells were used to set the background levels of fluorescence so that <1% of cells were determined as positive for any of the fluorochromes used. Cells incubated with single fluorochromes were used to verify detection of each individual fluorochromes. Colour compensation was carried out when false positive results were obtained. For example, if FL4 (APC) positive events were also observed in samples stained with a PerCP-Cy5.5 -conjugated antibody only, the flow cytometer settings were corrected – i.e. compensated – to show events as FL4 (APC) negative. This compensation was applied for each channel in order to avoid false positive results in the test samples, where dual staining was employed. Data was acquired with CellQuest software (BD Biosciences) and analysed with FlowJo software. The minimum number of events analysed for each sample was 10^5.

2.6.1 Surface staining of stimulated PBMC

After stimulation of PBMCs with appropriate pepmix for 10 days, PBMC were washed with 4 ml PBS containing 0.1% Bovine serum albumin [BSA] (FACS buffer) followed by centrifugation at 400 x g for 5 minutes. PBMC were then re-suspended in 50 μl FACS buffer, and transferred into 5 ml FACS tubes. Each tube contained 0.5×10^6 PBMC in a total volume of 50 μl. After addition of appropriate fluorochrome-conjugated monoclonal antibodies (mAb), cells were incubated on ice

for 20-30 minutes. Cells were then washed with FACS buffer, resuspended in 1 ml and analysed by flow cytometry. Control tubes were included in each immuno-staining experiment. These were an unstained sample control and appropriate single-colour compensation controls. The fluorochromes used were FITC, PE and APC. Thus, three different monoclonal antibodies with the same specificity (anti-human CD8 surface marker) were used, but labelled with the different fluorochrome, as follows: (i) anti-CD8-FITC, (ii) anti-CD8-PE and (iii) anti-CD8-APC. For more details see Table 1.1.

Table 2.1 Details of antibody surface staining of stimulated PBMC

Tube no	Tube Purpose	FITC	PE	APC
1	Unstained	none	none	none
2	FITC control	CD8 FITC	none	none
3	PE control	none	CD8 PE	None
4	APC control	none	none	CD8 APC
5	T cell subsets	CD8 FITC	CD3 PE	CD4 APC

All antibodies are mouse anti-human.

2.7 Intracellular cytokine staining (ICS)

PBMC (0.5×10^6) in 100 μl were aliquoted into the required number of tubes and 10 μl of individual peptide or 2 μl of pepmixes added (final peptide concentration 2 μg/ml). A control unstimulated PBMC tube was included, where no antigen was added to the cells. A further control was used where PBMC were stimulated with either PHA at final concentration of 10 μg/ml or Concanavalin A (ConA) [5 mg/ml] at final concentration of 5 μg/ml. The samples were mixed and incubated at 37°C, 5% CO_2 for 90 minutes. To prevent the export of cytokine, Brefeldin A (BFA) was added to a final concentration of 10 μg/ml. Tubes were then returned to the 37°C incubator for a further 14 hours incubation.

Cells were then harvested and washed with 4 ml FACS buffer, followed by centrifugation at 400 x g for 5 minutes. The cells were re-suspended in 50 µl FACS buffer before addition 1 µl per test of mAbs against CD8. The cells were then incubated on ice for 20 minutes. To fix the surface antibodies samples were washed by addition of 4 ml FACS buffer, followed by centrifugation at 400 x g for 5 minutes, before 100 µl fixation reagent BD Cytofix™ (4% paraformaldehyde in PBS) was added. Cells were then incubated in the dark for 15 minutes at RT. The cells were then washed with 4 ml FACS buffer followed by centrifugation at 400 x g for 5 minutes. Cells were then permeabilised by incubated for 5 minutes with 100 µl permeabilisation buffer, (eBioscience, UK). Without washing away the permeabilisation buffer, cells were incubated with anti fluorochrome-conjugated anti-cytokine mAb (anti-IFN-γ and anti-TNF-α) or isotype control antibodies for 30 minutes in the dark at RT (for more details of all mAbs used see Table 2.2). Finally, the cells were washed with 4 ml FACS buffer, centrifuged at 400 x g for 5 minutes, and resuspended in 400 µl FACS buffer, before being analysed on BD FACS Calibur flow cytometer.

Table 2.2 Details of antibody staining panel for ICS analysis of CD8 T cells

Tube no	Tube Purpose	FITC	PE	PC5
1	Unstained	none	none	none
2	FITC control	CD8 FITC	none	none
3	PE control	none	CD8 PE	None
4	PC5 Control	none	none	CD8 PC5
5	Isotype control	Mouse IgG FITC	Mouse IgG PE	CD8 PC5
6	Test Ag specificity	IFN-γ FITC	TNF-α PE	CD8 PC5
7	PHA control	IFN-γ FITC	TNF-α PE	CD8 PC5
8	Unstimulated PBMC	IFN-γ FITC	TNF-α PE	CD8 PC5

All antibodies are mouse anti-human.

2.8 Carboxy-Fluorescein Succinimidyl Ester (CFSE) proliferation assay

CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) was used to perform the CFSE labeling. Briefly, a stock solution of 5 mM CFSE was prepared by adding 18 µl DMSO (Component B) to one vial of CFSE (Component A). Five µl of CFSE solution was then added to $2x10^6$ PBMC (at a concentration of $1x10^6$ cells per ml), mixed gently and incubated for 10 minutes at 37°C. This was followed by washing three times with 10 ml PBS, with a 10 minute centrifugation at 400 x g between each wash. Viable cells were then recounted, and cells re-suspended in R10 growth media at a concentration of $1x10^6$ cells per ml in a well of 24-well plate. PBMC were then stimulated with the HHV6B-antigen Pepmixes (at final concentration of 2 µg/ml) or PHA (at final concentration of 10 µg/ml). Unstimulated PBMC were used as a negative control. Cells were then incubated for 7 days at 37°C.

On day 8, the cells were removed from the incubator and $0.5x10^6$ cells were aliquoted from the cell suspension into labelled FACS tubes. The cells were then washed with 4ml FACS buffer, and centrifuged at 400 x g for 5 minutes.. The supernatant was discarded and cells resuspended 50 µl of FACS buffer, before addition of 2.5 µg/ml of anti-CD8-APC mAbs followed by incubation on ice for 20 minutes. Unbound antibodies were removed by washing with 4ml FACS buffer and centrifuged at 400 x g for 5 minutes. Finally, cells were resuspended in 400 µl fixation reagent and mixed thoroughly by vortexing for 10-15 seconds. Flow cytometric analysis was done using BD FACS Calibur flow cytometer. CFSE was detected in the FL1 channel and CD8 in the FL4 channel.

2.9 Short-Term *in-vitro* T cell reactivation

Peptide-specific T cells were expanded *in-vitro* by incubation of PBMC with either pepmixes or individual peptides. PBMC (2×10^6) were pulsed with appropriate peptides at a final concentration of 10 μg/ml in 100 μl RPMI for 60 minutes at 37°C. Cells were then resuspended in 2 ml T cell media and seeded into a 24-well plate ($2x10^6$ cells per well). Cultures were supplemented on day 3 with 100 IU/ml IL-2 (Sigma, UK), which was replenished every 3 days. On day 10 T cells were harvested, counted and characterised by both functional and phenotypic approaches.

2.10 Enrichment of CD8+ T cells

Epitope mapping was performed using either CD8-enriched polyclonal T cell lines or in some cases total polyclonal T cell populations. CD8 enrichment was achieved by depletion of CD4-positive cells using an EasySep™ Human CD4 Positive Selection Kit (STEMCELL, Manchester, U.K.) according to the manufacturer's instructions. Briefly, cells were labelled with 10 μl EasySep® CD4 positive selection solution per 10^7 T cell lines for 15 minutes at RT, before 10 μl EasySep® magnetic nanoparticles were added and incubation continued for a further 10 minutes. The tube was then placed in magnet for 5 minutes, and CD8+ T cells collected by pouring off the supernatant into a fresh tube, with CD4 cells remain fixed in the original tube via the magnet. The efficiency of CD8+ T cell enrichment was analysed by flow cytometry staining the isolated cells with anti- CD4 and anti-CD8 mAb.

2.11 Enzyme-linked immunospot (ELISPOT) assay for single-cell IFN-γ release

Ninety-six well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were pre-coated with 15 μg/ml anti-IFN-γ mAb, 1-DIK for 3h in RT (MABTECH, Stockholm, Sweden). Cells were added to the wells at known cell numbers in duplicate or triplicate in the presence of pepmixes, peptide pools or individual peptides at a final concentration of 2 μg/ml. The plates were incubated overnight at 37°C in 5% CO_2. The cells were then discarded and a biotinylated anti-IFN-γ mAb, 7B6-1 (MABTECH), was added at 1 μg/ml and the plated incubated for 2-4 hours at RT. The wells were then washed 5 times with PBS containing 0.05% Tween 20 (PBS-T). Streptavidin-conjugated alkaline phosphatase (MABTECH) (1 mg/ml, diluted 1:1000 in PBS) 50 μl was then added to the wells, followed by incubation at RT for 2 hours. The wells were then washed 5 times with PBS-T, and individual IFN-γ-producing cells were detected as dark spots after addition of 100 μl 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (10 ul of each for every 1 ml of water at final concentration of 0.7 mmol/L) using an alkaline phosphatase-conjugated substrate kit (Bio-Rad, Richmond, CA). The numbers of spot forming cells (SFC) was then determined manually by counting under a dissection microscope.

2.12 IFN-γ Enzyme-Linked Immunosorbent Assay (ELISA)

Secretion of IFN-γ into culture supernatants during T cell assays was measured with a human IFN-γ ELISA Ready-Set-Go® set (eBioscience, UK), following the manufacturer's instructions. Briefly, 96-well Costar plates were coated with 100 μl capture antibody (anti-human IFN-γ MAb, diluted 1:250 with coating buffer) and the

plates incubated overnight at 4°C. The plate was then washed 5 times with PBS-T, and wells blocked for 1 hour with 200 µl assay diluent (10% bovine serum albumin in PBS). The blocking buffer was then removed without wash, and the standards and samples added to the plate at 100 µl/well. A total of 7 standards were prepared by 2-fold serial dilutions from the top standard which was 500 pg/ml. The plate was then incubated at RT for 2 hours, and then washed 5 times with PBS-T. After that, 100 µl of detection antibody (anti-human IFN-γ Biotin, diluted 1:250 with assay diluent) was added to each well. The plate was then incubated at RT for 1 hour, before being washed 5 times with PBS-T. Next, 100 µl Avidin-HRP (diluted 1:250 with assay diluent) was added to each well and the plate incubated at RT for 30 minutes. After washing 5 times with PBS-T, 100 µl 3, 3', 5, 5' tetramethyl benzidine (TMB) substrate solution was added to each well, with a further incubation of 15 minutes at RT in dark. Then, 50 µl stop solution (1M H_2SO_4) was added to each well. Finally, the absorbance was read at 450 nm using in Multiskan Spectrum microplate reader (Thermo Scientific) using SkanIt software 2.2. The concentration of IFN-γ (pg/ml) in each sample was calculated against the standard curve.

2.13 Identification of HLA restriction element

To determine the HLA restricting alleles of newly identified HHV6B peptide epitopes, autologous, partially HLA-matched and HLA mis-matched PBMC were used as targets in T cell assays. Target cells were loaded with individual peptides (2 µg/ml) for 2 hours in volume of 100 µl RPMI at 37°C, then washed 3 times with RPMI. Peptide-pulsed cells were then resuspended in R10 growth media at 1 x 10^5 cells/ml and used as targets in a T cell assay. 100 µl of target cells (1 x 10^4 cells) were added in

triplicate to wells of a 96-well V-bottom plate and 100 μl of effector T cells from short-term *in-vitro* reactivations were added at an Effector-to-Target (E:T) ratio of 10:1, 5:1 and 2.5:1.

The effectors and targets were co-cultured overnight at 37°C, before 100 μl of supernatant was harvested from each well and monitored for IFN-γ release using a Human IFN gamma ELISA Ready-SET-Go® kit (see section 2.13).

2.14 T cell recognition of HHV6B infected cells

To prepare targets cells infected by HHV6B for T cells assays, 2×10^6 PHA blasts in 500 μl T cell media were infected with 230 μl of HHV6B virus stocks HST or Z29 (Martin *et al.*, 2012) (section 2.5). Infections were incubated for 90 minutes at 37°C. A further 1.5 ml T cell media was then added. Six days after infection, the HHV6B wild-type virus-infected PHA blasts were used as targets for HHV6B-enriched antigen-specific T-cells. Specific T-cell recognition of targets was monitored by measuring release of IFN-γ in to the culture supernatant by ELISA. Infection by HHV6B was monitored visually for cytopathic effects and by immunofluorescence. T cells specific for each peptide to be tested were generated by stimulating PBMC with each individual peptide, and cultures were maintained for 10 days at 37°C, with IL2 being added to cultures from day 3. Polyclonal cultures were then used as effectors in virus recognition assays. Target cells incubated with effectors at an Effector-to-Target (E:T) ratio of 5:1 or 10:1. The effectors and targets were co-cultured overnight for 16h, before target cell recognition was monitored by measuring release of IFN-γ into the culture supernatant by ELISA. At E:T ratio 5:1, Virus infected PHA-blasts or mock

infected PHA-blasts (1×10^4 cells in 100ul media) were mixed with effector cells (5×10^4 cells in 100ul media) in 96-well V-bottom plate in triplicate. At E:T ratio 10:1, Virus infected PHA-blasts or mock infected PHA-blasts (1×10^4 cells in 100ul media) were mixed with effector cells (1×10^5 cells in 100ul media) in 96-well V-bottom plate in triplicate. At E:T ratio 20:1, Virus infected PHA-blasts or mock infected PHA-blasts (1×10^4 cells in 100ul media) were mixed with effector cells (2×10^5 cells in 100ul media) in 96-well V-bottom plate in triplicate.

For immunofluorescence monitoring of HHV6B-infected PHA blasts, cells were resuspended in 1 ml PBS at 1 x 10^6 cells/ml, 5 x10^4 cells in 50 μl were then added to defined areas on glass microscope slides (Shandon Cytospin 4 Cytocentrifuge; Thermo Scientific). After drying at RT, cells were fixed in acetone for 5 minutes at -20°C. Slides were incubated in pre-block (50 μl 5% normal goat serum in PBS) for 30 minutes, followed by pouring off excess block without washing. This was followed by incubation with 50 μl mouse-anti–human HHV6B mAb (monoclonal antibody MAB8535, clone C3108-003; Chemicon) diluted 1:50 in PBS for 1 hour at 37°C in 5% CO_2. Slides were then washed 5 times in PBS for 2 minutes/wash, 50 μl secondary antibody (goat anti-mouse IgG-FITC diluted 1:200 in PBS) was then added to each cell spot and incubated for 30 minutes. The slide was then washed 5 times in PBS. Finally, slides were mounted by Vectashield DAPI then analysed with a Nikon fluorescence microscope. For each HHV6B-infected PHA blasts stained by each immunofluorescence two negative controls were added. Firstly, an isotype control was used to ensure the specificity of binding of the primary antibody to the antigen. A second negative control was used, where no primary antibody was added.

2.15 Statistical Analysis

Graphpad Prism version 5 software was used for all statistical tests. The Mann Whitney non-parametric test was used to compare differences between sample groups. (Downloaded from http://www.graphpad.com/prism/Prism.htm.).

Chapter 3

3. *Ex-vivo* analysis of T cell responses to HHV6B in healthy donors

When this project was initiated studies looking at the T cell responses to HHV6 were sparse. HHV6-specific T cell lines and T cell clones had been established for CD4+ T cells, however there was no data on the CD8+ T cell response to HHV6 (Yakushijin *et al.*, 1991, 1992; Yasukawa *et al.*, 1993; Koide *et al.*, 1998; Tejada-Simon *et al.*, 2002; Kumagai *et al.*, 2006). Thus, this was a neglected area of herpesvirus immunology and, with the advances in technology available for analysing human immune responses over the last 15-20 years, warranted renewed investigation. The first objective was to utilize a panel of healthy donors and investigate if T cell responses to HHV6 could be detected and characterised directly *ex-vivo*, focusing on CD8+ T cell responses.

Previously a study had been carried out using putative HLA-A2 binding peptides derived from the HHV6 U90 antigen, predicted using the SYFPEITHI epitope prediction programme (Rammensee *et al.*, 1999), and ELISpot, had failed to identify any HHV6 CD8+ T cell responses in a group of 14 healthy donors [Naeem Khan, personal communication]. Thus, the approach to be employed in this thesis was to focus on a wider panel of HHV6 antigens, which are functionally equivalent to known strongly immunogenic HCMV antigens, on the basis that these would also be immunogenic in HHV6. The HHV6 antigens to be investigated were U11, U39, U54 and U90, which are equivalent to the immunogenic HCMV antigens pp150, gB, pp65 and IE1 (see Table 3.1) (Efstathiou *et al.*, 1988; Lawrence *et al.*, 1990; Martin *et al.*, 1991; Nicholas, 1994; Gompels *et al.*, 1995; Khan, 2007). A broad approach was taken, whereby pepmixes, single tube libraries of 15-mer peptides overlapping by

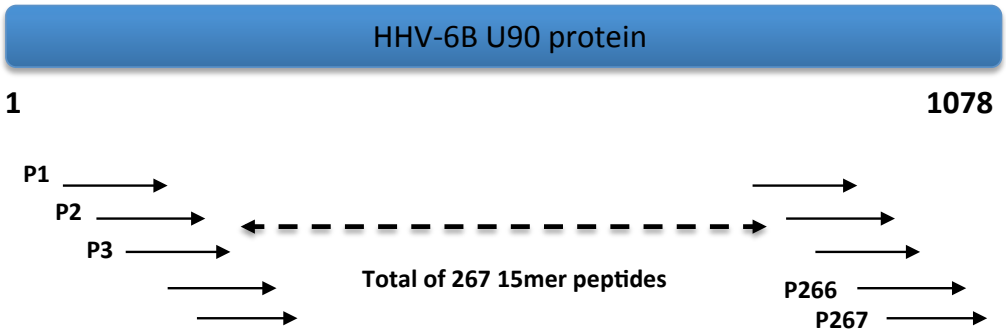
11aa (for a schematic representation see Figure 3.1), representing the HHV6 antigens U11, U39, U54 and U90 were used to stimulate PBMC, before analysis of T cell responses. Using single tube libraries of overlapping 15-mer peptides is a powerful tool for an unbiased screen for global CD8+T cells responses in a single reaction (Kern *et al.*, 2000). As HHV6B is the most common virus subtype, present in more than 90% of HHV6-infected individuals (Levy *et al.*, 1990), pepmixes used were based on the amino acid sequence of this strain of the virus. HHV6B antigen-specific T cell responses were monitored by two different approaches, (i) ICS for both IFN-γ and TNF-α and (ii) CFSE proliferation assays, as outlined in Figure 3.2.

Table 3.1 HHV6B proteins analysed in this thesis, their equivalent HCMV protein and their properties*

HHV6B Protein	HCMV Protein	Properties
U11	pp150 (UL32)	Antigenic tegument protein
U39	gB (UL55)	Major glycoprotein
U54	pp65 (UL83)	Tegument protein virion transactivator
U90	IE1 (UL123)	Immediately early transactivator

*For HHV6B the open reading frame nomenclature is used, for HCMV the functional gene name is used with the relevant open reading frame in brackets.

A total of 30 healthy donors were analysed by these approaches. The HHV6 status of the donors was not known, however based on the estimated worldwide prevalence of HHV6 of greater than 90%, it was assumed that the majority of donors would be positive (Okuno *et al.*, 1989; Levy *et al.*, 1990; Braun *et al.*, 1997; Zerr *et al.*, 2005). The HCMV status of donors was confirmed by ELISA. The HLA type was available for the majority of donors. Full details of donors used throughout this thesis are presented in the Appendix (Appendix Table 8.1).



Representation of peptides overlap

```
P1   MESAKDTTSTSMFIL
P2       KDTTSTSMFILGKPS
P3            STSMFILGKPSGNNM
```

Figure 3.1 Schematic representation of a whole protein spanning pepmix for HHV6B U90.

The U90 protein is 1078 aa in length, and the single tube pepmix contains a total of 267 15-mer peptides, overlapping by 11 amino acids, spanning the entire protein sequence. The peptides are represented by black arrows, and the first three peptides (P1-P3) are shown in full to demonstrate the overlaps.

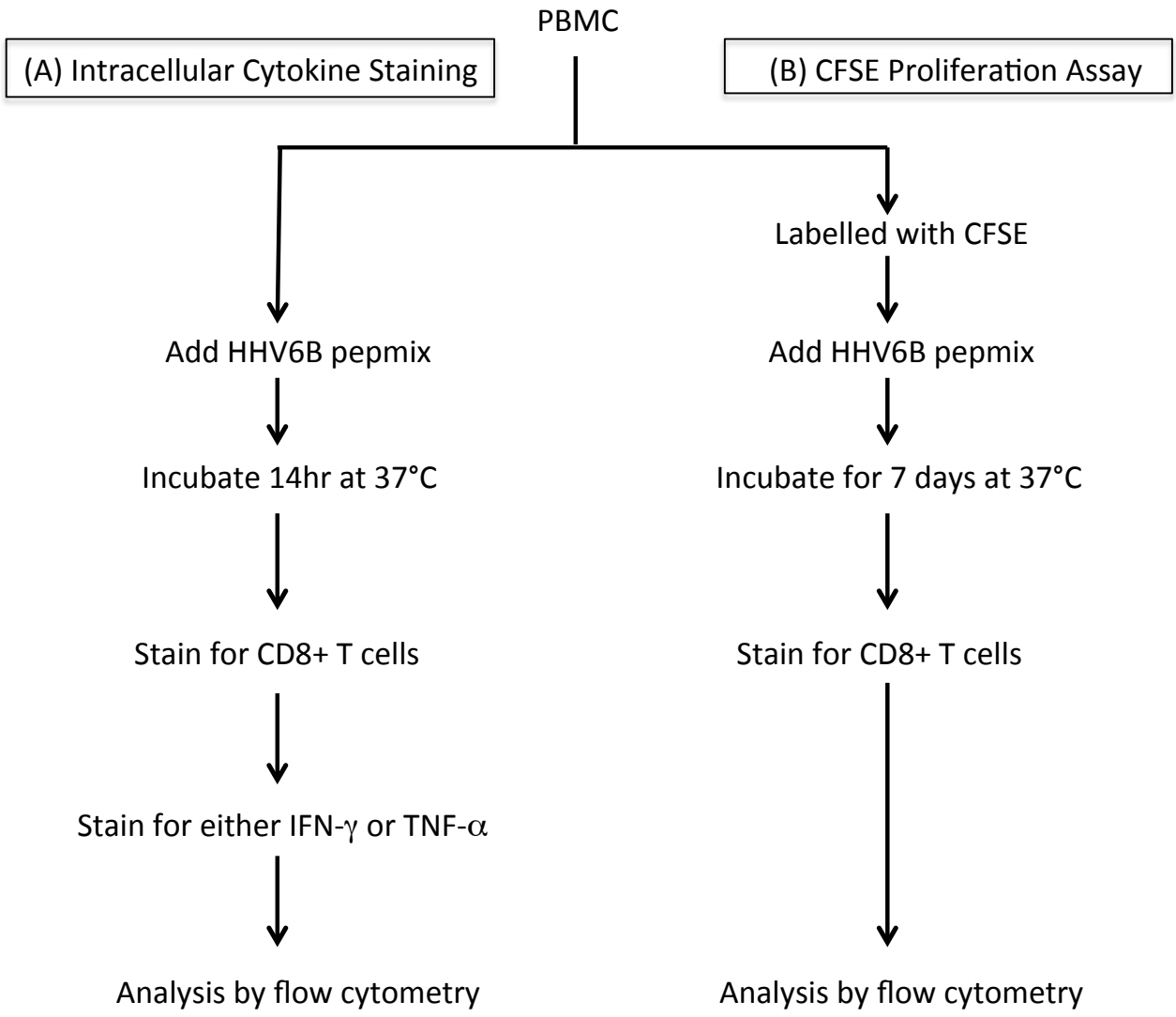


Figure 3.2 Flowchart showing the approaches used for *ex-vivo* analysis of T cell responses to HHV6B antigens.

PBMC were either (A) incubated directly with HHV6B antigen pepmixes before being stained for CD8+ T cells, and either IFN-γ or TNF-α then analysed by flow cytometry, or (B) labelled with CFSE, before being incubated with HHV6B antigen pepmixes, proliferation of T cells was analysed by staining for CD8+ T cells by flow cytometry.

3.1 Analysis of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90

In an initial attempt to determine the frequency of HHV6B antigen-specific CD8+ T cells directly in PBMC, ICS was used to investigate production of both IFN-γ and TNF-α after stimulation with HHV6B pepmixes for U11, U39, U54 and U90. All experiments were carried out using fresh PBMC, which were stimulated overnight with individual pepmixes before being stained with fluorescently labelled anti-CD8 and either anti-IFN-γ or anti-TNF-α mAbs. In addition, HCMV+ve donors were also stimulated with pepmixes representing HCMV antigens pp65, gB, IE1 and IE2, to give an indication of the ability of T cells to respond to antigenic stimuli. ConA stimulated PBMC was used as positive control throughout, and unstimulated PBMC as the negative control. Subsequently, the ability of HHV6B antigen pepmixes to stimulate CD8+ T cell proliferation was investigated by CSFE proliferation assays (Lyons and Parish, 1994).

3.1.1 Detection of HHV6B antigen-specific IFN-γ positive, CD8+ T cells by intracellular cytokine staining

Thirty healthy donors were analysed for IFN-γ+ve, CD8+ responses to HHV6B U11, U39, U54 and U90, and data presented as percentage of antigen-specific CD8+ IFNγ–producing T cells in total CD8+ lymphocytes. A positive result in this study was considered as a value of at least 0.02% above the negative control value (Mesa *et al.*, 2010; Gómez *et al.*, 2011). Representative examples of the CD8+ T cell staining profiles are shown for donors HD01 and HD05 in Figure 3.3. The flow cytometry

plots of ICS in the left hand panel shows the CD8+ T cell response from healthy donor HD01. Overall responses detected in donor HD01 were very low, ranging from 0% for U11, 0.01% for U90, 0.02% for U39 to 0.04% for U54. The background level for unstimulated PBMC was 0.01%, and based on the criteria stated above only the response to U54 was considered positive. In the same donor responses to the immunogenic HCMV antigens pp65 and IE1 were 0.12% and 1.54% respectively, suggesting that the equivalent antigens in HHV6 were much less immunogenic. ICS analysis for PBMC from donor HD05 stimulated with the same antigens is shown in the right hand panels (Figure 3.3). Again, overall responses detected against HHV6B antigens in this donor were very low, ranging from 0.03% for U11, U39 and U54 to 0.16% for U90. The response present in unstimulated cells was 0.03%, indicated that this donor has a CD8+ T cell response to HHV6B U90. HD05 was shown to contain strong responses to HCMV pp65 (0.83%) and IE1 (1.54%) (Figure 3.3), reinforcing the observation from donor HD01 that HHV6 is significantly less immunogenic than HCMV.

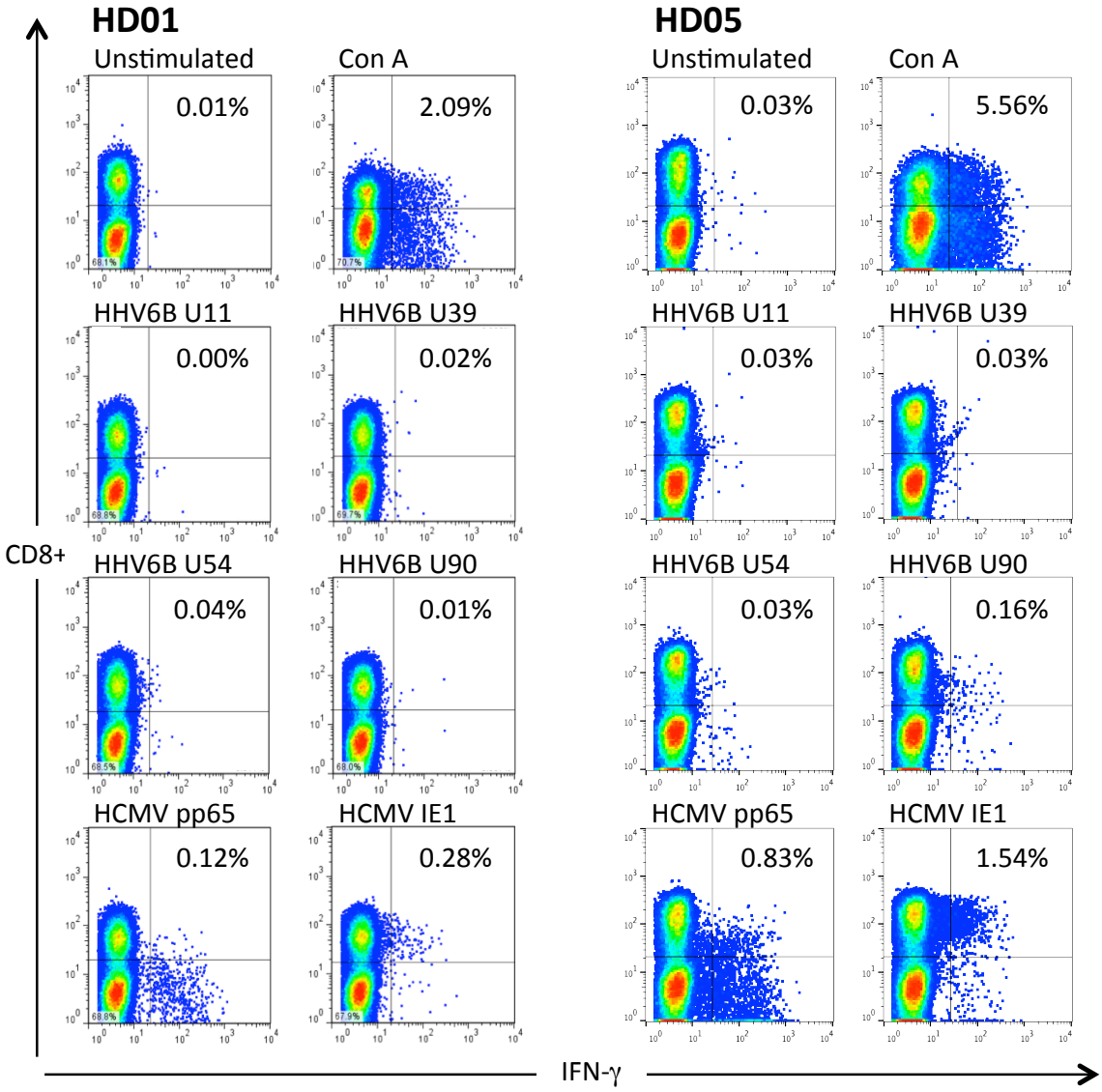


Figure 3.3 Analysis of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in donor HD01 and HD05 *ex-vivo* by intracellular cytokine staining for IFN-γ

PBMC ($5x10^5$) from donors HD01 and HD05 were stimulated with HHV6B whole antigen pepmixes for U11, U39, U54 and U90, for 16 hours before being analysed for production of IFN-γ by ICS. PBMC were stimulated with Concanavalin A (Con A) as a positive control, and also with pepmixes representing HCMV pp65 and IE1. Unstimulated PBMC served as a negative control. Values indicated in upper right quadrants denote the percentage of CD8+ IFN-γ producing cells out of total CD8+ T cells. Data was collected on a BD FACSCalibur and analysed using FlowJo software (Treestar).

A further 28 donors were analysed in this way, and the percentages of CD8+ IFN-γ producing T cells specific for the HHV6B antigens tested are shown in Table 3.2. Overall, using the 0.02% cut off criteria 13 donors were found to have responses directed against HHV6B antigens. However, in general, as seen in Figure 3.3 for donors HD01 and HD05, responses were very low. For example, donor HD02 responded U54 and U90 (0.04% and 0.08% respectively, with a background of 0.02%), and donor HD20 responded to U90 (0.18%, with a background of 0.02%). The mean frequency of CD8+ T cells producing IFN-γ in response to U11 was 0.006% of total CD8+ T cells in peripheral blood, with maximum response of 0.04% (after subtraction of the background for the negative control). The mean frequency against U39 was 0.006% of total CD8+ T cells, with a maximum response of 0.03%. The mean frequency against U54 was 0.01% of total CD8+ T cells, with a maximum response of 0.04%. Finally, the mean frequency against U90 was 0.03% of total CD8+ T cells, with a maximum response of 0.20% (Figure 3.4). This was in direct contrast to responses detectable against HCMV, for example individual responses detected against IE1 ranged from 0.02 to 8.74 % (Full data for all donors tested is shown in Appendix Table 8.2). The means frequencies detected against the HCMV antigens are shown in Figure 3.4. Overall, as analysed by ICS for IFN-γ production, 13% of donors responded to HHV6B U11, 10% to U39, 30% to U54 and 33% to U90 (Figure 3.9 and Table 3.5).

Table 3.2 Total data for CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in 30 donors analysed *ex-vivo* by intracellular cytokine staining for IFN-γ*

Donor	U11	U39	U54	U90	Neg**	Pos***
HD01	0.00	0.02	0.04	0.01	0.01	2.09
HD02	0.01	0.02	0.04	0.08	0.02	4.03
HD03	0.03	0.02	0.04	0.04	0.03	11.18
HD04	0.00	0.02	0.02	0.01	0.02	1.26
HD05	0.03	0.03	0.03	0.16	0.03	5.56
HD06	0.01	0.02	0.02	0.00	0.02	0.05
HD07	0.04	0.03	0.05	0.05	0.04	0.14
HD08	0.06	0.06	0.04	0.10	0.05	0.30
HD09	0.02	0.03	0.05	0.21	0.02	1.27
HD10	0.03	0.03	0.03	0.03	0.01	2.68
HD11	0.04	0.04	0.04	0.04	0.02	3.54
HD12	0.04	0.05	0.08	0.03	0.04	4.36
HD13	0.08	0.04	0.07	0.19	0.05	2.73
HD14	0.05	0.04	0.03	0.06	0.01	3.07
HD15	0.02	0.03	0.03	0.01	0.03	2.62
HD16	0.02	0.01	0.01	0.02	0.01	0.72
HD17	0.15	0.09	0.06	0.05	0.18	0.47
HD18	0.10	0.09	0.10	0.09	0.10	0.97
HD19	0.00	0.02	0.02	0.01	0.03	0.20
HD20	0.04	0.05	0.01	0.18	0.04	0.63
HD21	0.04	0.02	0.02	0.03	0.06	1.99
HD22	0.01	0.02	0.00	0.02	0.01	0.22
HD23	0.03	0.02	0.02	0.02	0.02	0.85
HD24	0.02	0.02	0.00	0.02	0.01	0.92
HD25	0.00	0.00	0.00	0.01	0.00	0.44
HD26	0.00	0.01	0.02	0.01	0.00	0.11
HD27	0.00	0.00	0.00	0.00	0.01	0.38
HD28	0.01	0.00	0.00	0.00	0.02	2.59
HD29	0.00	0.00	0.01	0.01	0.00	2.17
HD30	0.00	0.00	0.01	0.12	0.00	2.38
Total = 13	4	3	9	10		

*Values indicated are the percentage of CD8+ T cells producing IFN-γ. **Unstimulated PBMC served as a negative control. ***Stimulated with Concanavalin A served as a positive control. Numbers in the final row indicate numbers of positive donors.

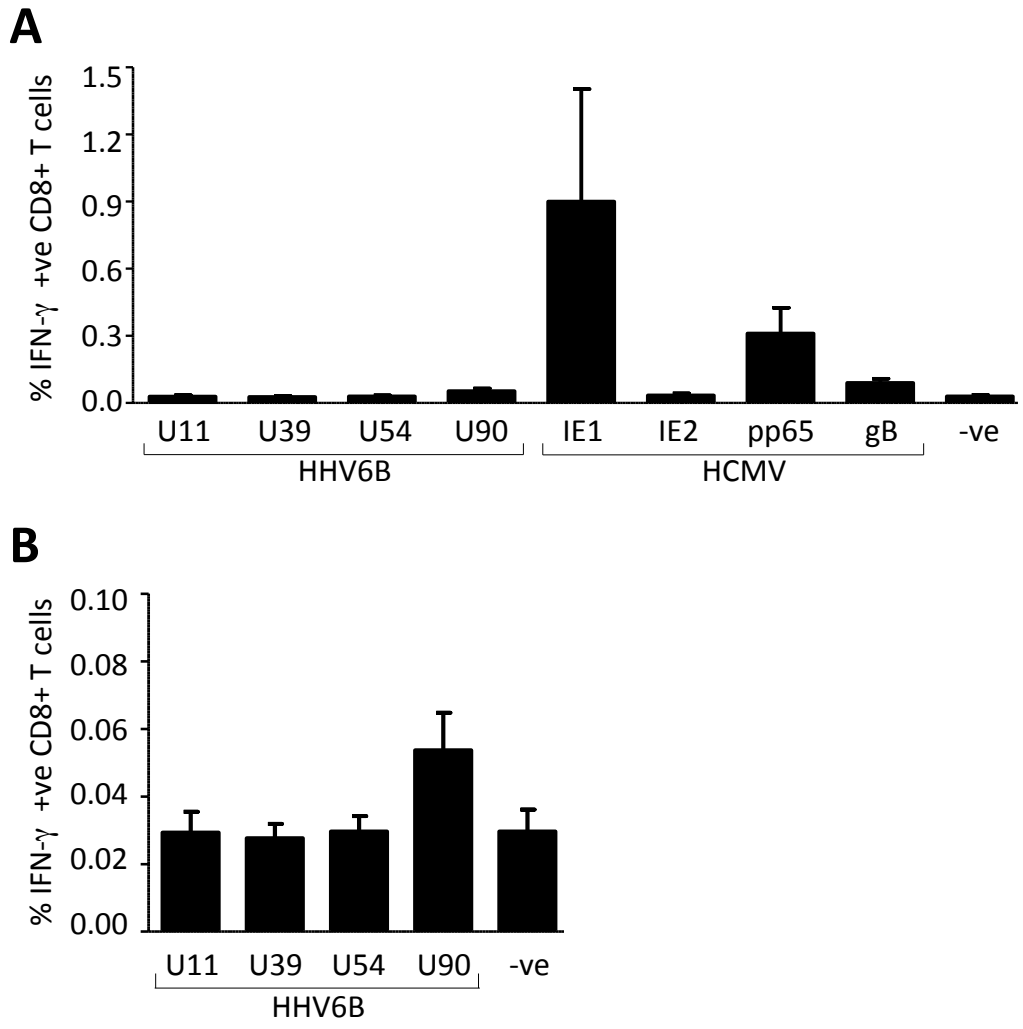


Figure 3.4 Overall CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 and HCMV antigens IE1, IE2, pp65 and gB analysed *ex-vivo* by intracellular cytokine staining for IFN-γ

(A) Summary of the donor data for the ICS analysis of CD8+ IFN-γ responses to HHV6B antigens U11, U39, U54 and U90 (n=30), HCMV antigens IE1, IE2, pp65 and gB (n=17) and –ve control (n=30). The results are expressed as mean value of CD8+ cells producing IFN-γ ± SEM. Unstimulated PBMC were used as a negative control (-ve). (B) The data presented in part (A) for HHV6B is shown here on its own to expand the y-axis.

3.1.2 Detection of intracellular TNF-α positive, CD8+ T cells by intracellular cytokine staining.

As the CD8+ T responses to HHV6B antigens as detected by ICS for IFN-γ were very low, it was possible that analysing for another effector cytokine might reveal stronger responses, as measuring of one cytokine may not reflect the actual level of the T cell responses (Nebbia *et al.*, 2008). Thus, the same group of donors were analysed for TNF-α positive CD8+ T cells after stimulation with the same HHV6B antigen pepmixes, and HCMV controls (pp65 and gB). A representative example of ICS staining for HHV6B-specific TNF-α positive CD8+ T cells for donor HD05 is shown in Figure 3.5 (left hand panel). Similar to IFN-γ responses, TNF-α +ve CD8+ T cells against the HHV6B antigens were detected at a very low frequency. For donor HD05 responses ranged from 0.03% for U11, to 0.04% for U54, to 0.05% for U39 and 0.22% for U90. With a background level of 0.03%, it was determined that HD05 had detectable TNF-α +ve CD8+ T cell responses against HHV6B antigens U39 and U90. In comparison, the frequency of TNF-α +ve CD8+ T cells against HCMV pp65 was 0.86% and gB 3.91%.

Another example is shown for donor HD16 in Figure 3.5 (right hand panel) for TNF-α against the HHV6B antigens and control antigens (HCMV pp65 and gB). HD16 did not have any detectable CD8+ T cell response for TNF-α against the HHV6B antigens.

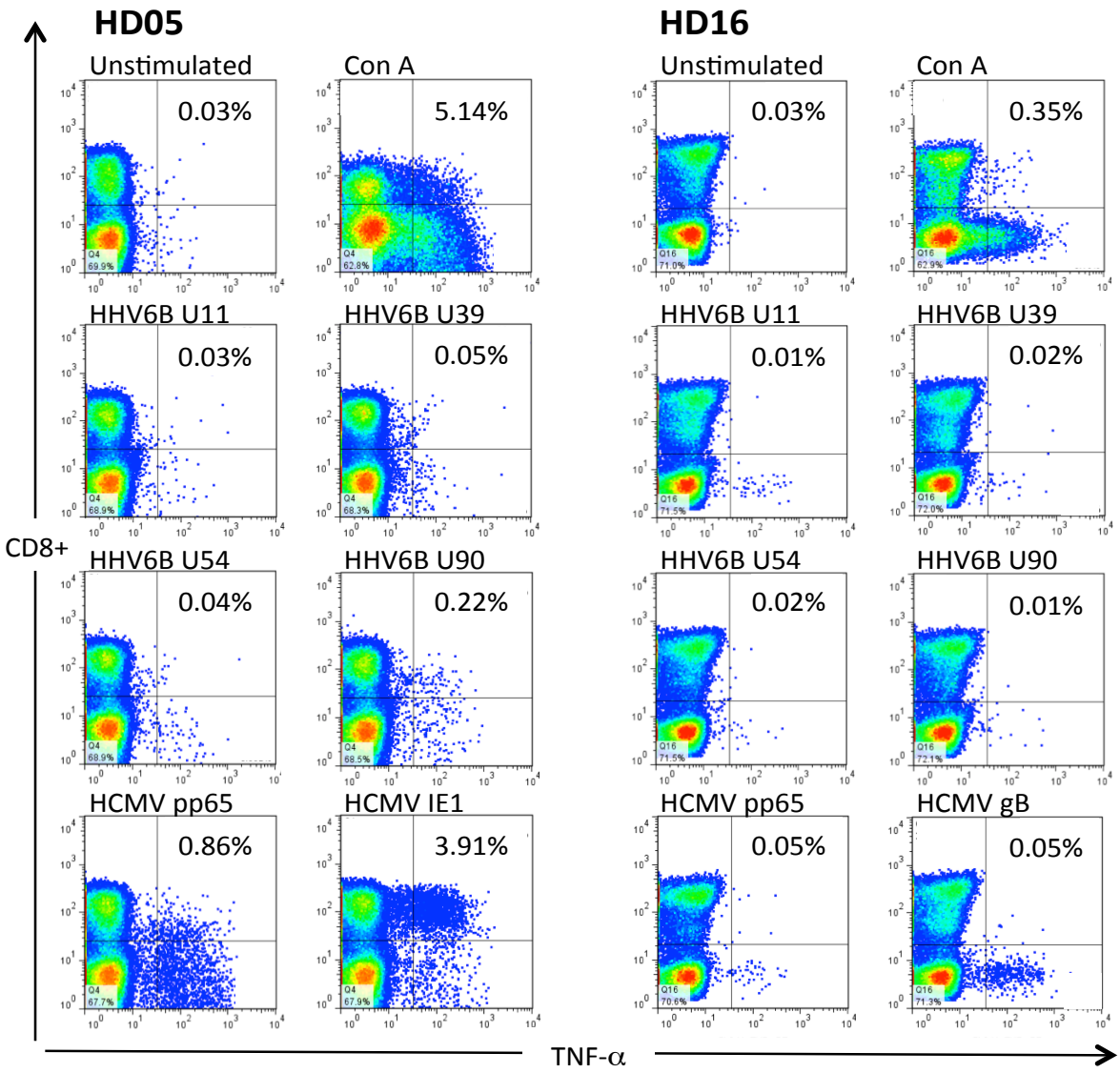


Figure 3.5 Analysis of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in donor HD05 *ex-vivo* by intracellular cytokine staining for TNF-α

PBMC ($5x10^5$) from donors HD05 were stimulated with HHV6B whole antigen pepmixes for U11, U39, U54 and U90 for 16 hours, before being analysed for production of TNF-α by ICS. PBMC were stimulated with Concanavalin A (Con A) as a positive control, and also with pepmixes representing HCMV pp65, IE1 or gB. Unstimulated PBMC served as a negative control. Values indicated in upper right quadrants denote the percentage of CD8+ TNF-α producing cells out of total CD8+ T cells. Data was collected on a BD FACSCalibur and analysed using FlowJo software (Treestar).

The percentages of CD8+ TNF-α producing T cells specific for the HHV6B antigens tested in all donors analysed are shown in Table 3.3. Again, as seen for IFN-γ, responses were very low in all donors. Overall, using the 0.02% cut off criteria 20 donors were found to have responses directed against HHV6B antigens. For example, donor HD02 responded to U54 and U90 (0.06% and 0.12% respectively, with a background of 0.02%), and donor HD13 responded U90 (0.14%, with a background of 0.08%). The mean frequency of CD8+ T cells producing TNF-α in response to U11 was 0.007% of total CD8+ T cells in peripheral blood, with maximum response of 0.04%. The mean frequency against U39 was 0.01% of total CD8+ T cells, with a maximum response of 0.07%. The mean frequency against U54 was 0.02% of total CD8+ T cells, with a maximum response of 0.08%. Finally, the mean frequency against U90 was 0.04% of total CD8+ T cells, with a maximum response of 0.19% (Figure 3.6). Although U90 was the highest response of HHV6B antigen, however the difference for both U90 and U54 were significant when compared with the negative control (Figure 3.6B). This was in direct contrast to responses detectable against HCMV, for example against IE1 responses ranged from 0.03 to 8%. (Full data for all donors tested is shown in Appendix Table 8.3). The means frequencies detected against the HCMV antigens are shown in Figure 3.6. Overall, as analysed by ICS for TNF-α production, 20% of donors responded to HHV6B U11, 40% to U39, 30% to U54 and 50% to U90 (Figure 3.9 and Table 3.5).

Table 3.3 Total data for CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in 30 donors analysed *ex-vivo* by intracellular cytokine staining for TNF-α*

Donor	U11	U39	U54	U90	Neg**	Pos***
HD01	0.04	0.05	0.09	0.04	0.03	4.48
HD02	0.02	0.02	0.06	0.12	0.02	2.68
HD03	0.06	0.05	0.11	0.12	0.03	6.65
HD04	0.04	0.04	0.09	0.02	0.06	2.75
HD05	0.03	0.05	0.04	0.22	0.03	5.14
HD06	0.06	0.11	0.03	0.09	0.06	4.86
HD07	0.09	0.12	0.13	0.12	0.06	3.77
HD08	0.07	0.05	0.10	0.11	0.07	3.37
HD09	0.02	0.05	0.06	0.16	0.02	0.73
HD10	0.05	0.05	0.05	0.04	0.05	1.50
HD11	0.06	0.04	0.02	0.11	0.02	1.14
HD12	0.03	0.02	0.06	0.04	0.03	4.25
HD13	0.08	0.08	0.08	0.14	0.08	1.64
HD14	0.08	0.08	0.06	0.09	0.05	1.21
HD15	0.06	0.06	0.08	0.07	0.06	1.56
HD16	0.01	0.02	0.02	0.01	0.03	0.35
HD17	0.07	0.02	0.05	0.00	0.09	0.28
HD18	0.03	0.06	0.07	0.20	0.07	2.70
HD19	0.02	0.01	0.02	0.04	0.02	1.57
HD20	0.02	0.08	0.10	0.12	0.07	3.85
HD21	0.02	0.02	0.03	0.03	0.03	1.33
HD22	0.02	0.03	0.03	0.02	0.01	1.25
HD23	0.02	0.01	0.02	0.02	0.02	0.19
HD24	0.04	0.04	0.00	0.04	0.02	0.86
HD25	0.03	0.02	0.01	0.02	0.03	2.20
HD26	0.03	0.04	0.03	0.02	0.02	0.16
HD27	0.02	0.03	0.01	0.00	0.04	0.29
HD28	0.02	0.04	0.01	0.02	0.03	2.17
HD29	0.02	0.01	0.02	0.02	0.02	2.45
HD30	0.03	0.02	0.04	0.03	0.00	1.48
Total = 20	6	9	12	15		

*Values indicated are the percentage of CD8+ T cells producing TNF-α. **Unstimulated PBMC served as a negative control. ***Stimulated with Concanavalin A served as a positive control. Numbers in the final row indicate numbers of positive donors.

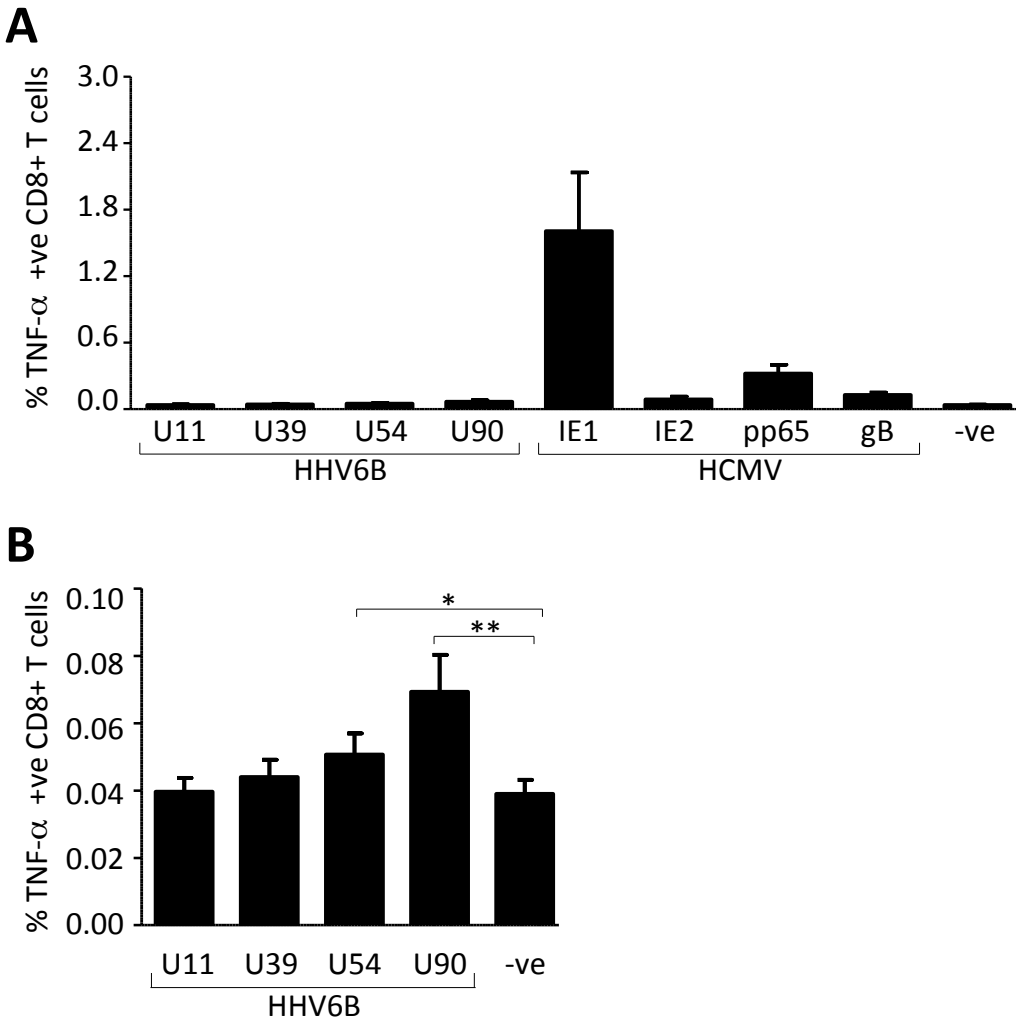


Figure 3.6 Overall CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 and HCMV antigens IE1, IE2, pp65 and gB analysed *ex-vivo* by intracellular cytokine staining for TNF-α

(A) Summary of the donor data for ICS analysis of CD8+ TNF-α+ responses to HHV6B antigens U11, U39, U54 and U90 (n=30), HCMV antigens IE1, IE2, pp65 and gB (n=17) and –ve control (n=30). The results are expressed as mean value of CD8+ cells producing TNF-α ± SEM (error bars). Unstimulated PBMC were used as a negative control (-ve) and stimulated with Concanavalin A as a positive control (+ve). (B) The data presented in figure A for HHV6B is shown here on its own to expand the y-axis. Statistical differences (**p<0.008 and *p<0.05) between different groups were calculated by Wilcoxon test.

3.1.3 Detection of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 by CFSE proliferation assays

Since it was clear that the responses detected by ICS for both IFN-γ and TNF-α were very weak, CFSE proliferation assays were used to investigate if HHV6B antigen induced cell proliferation was more readily detectable. Eleven donors were tested for CD8+ proliferation in response to HHV6B antigens U11, U39, U54 and U90. An example of a CSFE proliferation assay for donor HD09 is shown in Figure 3.7. Values indicated in upper left quadrants denote the percentage of proliferated CD8+ T cells out of total CD8+ T cells. A positive result in this study was considered as a value of at least 0.5% above the negative control value (ProImmune, 2014). Overall responses detected in donor HD09 were very low, ranging from 0.65% for U11, 0.7% for U39, 0.82% for U54 to 2.47% for U90. The background level for unstimulated PBMC was 0.47%, and based on the criteria stated above only the response to U90 was considered positive.

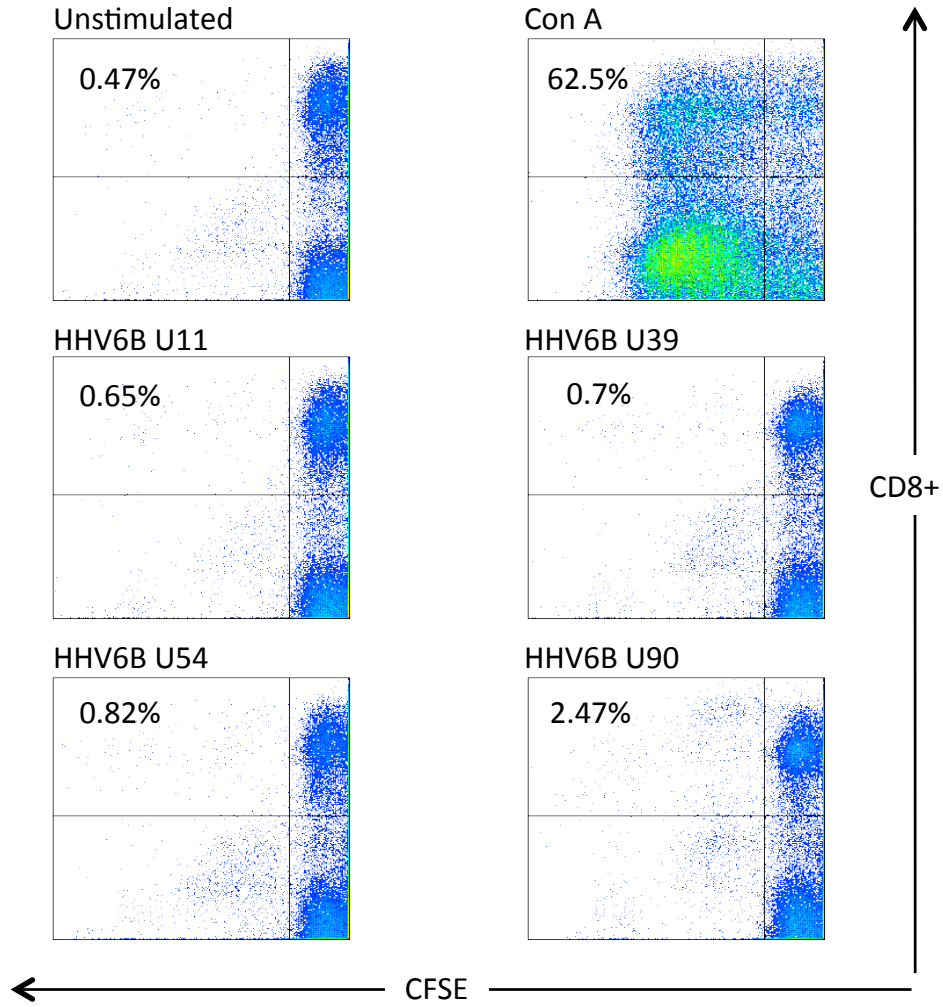


Figure 3.7 Analysis of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in donor HD09 *ex-vivo* by CFSE proliferation assay

PBMC were isolated from donor HD09 and $2x10^6$ were labelled with 5µM of CFSE for 10 minutes at 37°C then stimulated with HHV6B whole antigen pepmixes for U11, U39, U54 and U90 for 7 days then labelled with anti-CD8+ for 20 minutes on ice and analysed on a flow cytometry. PBMC were stimulated with Concanavalin A (Con A) as a positive control. Unstimulated PBMC served as a negative control. Values indicated in upper left quadrants denote the percentage of proliferated CD8+ T cells out of total CD8+ T cells. Data was collected on a BD FACSCalibur and analysed using FlowJo software (Treestar).

A further 10 donors were analysed in this way, and the percentages of CD8+ T cell proliferation responses to HHV6B antigens tested in all donors analysed are shown in Table 3.4. Overall, using the 0.5% cut off criteria 5 donors were found to have responses directed against HHV6B antigens. Table 3.5 in section 3.1.4 shows which donors were positive and what antigens they responded to. However, in general, as seen in Figure 3.7 for donor HD09, responses were very low. For example, donor HD02 responded U54 and U90 (2.36% and 3.59% respectively, with a background of 1.7%). The mean frequency of proliferated CD8+ T cells in response to HHV6B U11 was 0.09% of total CD8+ T cells in peripheral blood, with maximum response of 0.51%. The mean frequency against U39 was 0.09% of total CD8+ T cells, with a maximum response of 1.3%. The mean frequency against U54 was 0.36% of total CD8+ T cells, with a maximum response of 0.66%. Finally, the mean frequency against U90 was 0.45% % of total CD8+ T cells, with a maximum response of 1.89%. (Figure 3.8A). This was in direct contrast to responses detectable against the positeve control, which ranged from 37.3 to 80.9%. Overall, as analysed by CFSE proliferation assay, 9% of donors responded to HHV6B U11, 9% to U39, 36% to U54 and 27% to U90 (Figure 3.9).

Table 3.4 Total data for CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in 11 donors analysed *ex-vivo* by CFSE proliferation assay*

Donor	U11	U39	U54	U90	Neg**	Pos***
HD01	1.55	2.80	2.00	1.94	1.5	60.5
HD02	1.33	1.49	2.36	3.59	1.7	70.7
HD03	2.18	2.07	2.19	2.37	1.67	78.8
HD07	0.06	0.08	0.11	0.11	0.09	73.30
HD09	0.65	0.70	0.82	2.47	0.47	62.50
HD14	1.03	0.95	1.19	1.08	0.75	80.90
HD16	2.75	2.32	3.06	2.29	2.45	45.1
HD17	0.59	1.05	1.43	0.65	1.17	54.7
HD18	0.63	0.60	0.65	0.51	0.99	45.7
HD19	1.08	1.49	1.30	1.38	1.28	37.3
HD21	4.62	4.31	4.25	4.77	4.99	61.4
Total = 5	1	1	4	3		

*Values indicated are the percentage of proliferated CD8+ T cells. **Unstimulated PBMC served as a negative control. ***Stimulated with Concanavalin A served as a positive control. Numbers in the final row indicate numbers of positive donors.

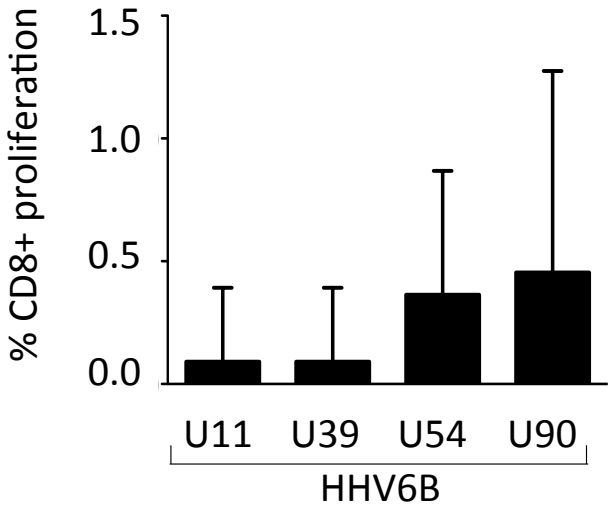


Figure 3.8 Summary of CD8+ T cell responses to HHV6B antigens U11, U39, U54 and U90 in 11 donors analysed *ex-vivo* by CFSE proliferation assay

A total of 11 donors were analysed by CFSE proliferation assay for: HHV6B antigens U11, U39, U54 and U90. The results are expressed as mean value of proliferated CD8+ T cells ± SEM after subtract the negative control from the individual reading. Unstimulated PBMC were used as a negative control.

3.1.4 Summary of CD8+ T cell responses to HHV6B detected *ex-vivo* in PBMC

A complete summary of the *ex-vivo* CD8+ responses against U11, U39, U54 and U90 as detected by ICS (for IFN-γ and TNF-α) and CSFE staining are shown in Table 3.5. As stated, *ex-vivo* responses detected against the HHV6B antigens tested in this chapter were very weak. Despite this, the following conclusion could be made. Using ICS and detection of IFN-γ production, a total of thirteen donors (43%) responded to HHV6B antigens, as follows: four donors (HD10, HD11, HD13 and HD14) responded to U11; three donors (HD10, HD11 and HD14) responded to U39; nine donors (HD01, HD02, HD09, HD10, HD11, HD12, HD13, HD14 and HD26) responded to U54 and ten donors (HD02, HD05, HD08, HD09, HD10, HD11, HD12, HD13, HD14, HD20 and HD30) responded to U90. Using ICS and detection of TNF-α production a total of twenty donors (67%) responded to HHV6B antigens as follows: six donors (HD03, HD07, HD11, HD14, HD24 and HD30). responded to U11; nine donors (HD01, HD03, HD05, HD06, HD07, HD09, HD11, HD14 and HD28) responded to U39; twelve donors (HD01, HD02, HD03, HD04, HD07, HD08, HD09, HD12, HD15, HD20, HD29 and HD30) respond to U54 and fifteen donors (HD02, HD03, HD05, HD06, HD07, HD08, HD09, HD11, HD13, HD14, HD18, HD19, HD20, HD29 and HD30) responding to U90. When the percentage of donors responding via IFN-γ or TNF-α is compared, although more donors responded based on TNF-α measurement, the overall hierarchy is generally similar. Based on IFN-γ production, 33% of donors responded to U90, 30% to U54, 13% to U11 and 10% to U39. Whereas, based on TNF-α production 50% of donors responded to U90, 40% to U54, 30% to U39 and 20% to U11 (Figure 3.9).

With U90 being the most immunogenic for both secretion of IFN-γ and TNF-α, followed by U54. For IFN-γ, this is followed by U11 and U39, whereas this is reversed for TNF-α.

Using CFSE proliferation assay five out of eleven donors (45%) responded to the antignens tested, generally agreeing with the data for ICS. Although the number of donors tested was small, using this assay U54 was the most immunogenic with four donors (36%) responding (HD01, HD02, HD03 and HD16), with three donors (27%) responding to U90 (HD02, HD03 and HD09), and one donor (9%) responding to U11 (HD03) and one donor (9%) to U39 (HD01). In general agreeing with the hierarchy determined using ICS (Figure 3.9).

Table 3.5 Summary of 24 positive CD8+ T cell responses to HHV6B detected *ex-vivo* in PBMC

Donor	IFN-γ	TNF-α	Proliferation
HD01	U54	U39 and U54	U39 and U54
HD02	U54 and U90	U54 and U90	U54 and U90
HD03	NR*	All antigens	U11, U54 and U90
HD04	NR	U54	NT**
HD05	U90	U39 and U90	NT
HD06	NR	U39 and U90	NT
HD07	NR	All antigens	NR
HD08	U90	U54 and U90	NT
HD09	U54 and U90	U39, U54 and U90	U90
HD10	All antigens	NR	NT
HD11	All antigens	U11, U39 and U90	NT
HD12	U54	U54	NT
HD13	U11, U54 and U90	U90	NT
HD14	All antigens	U11, U39 and U90	NR
HD15	NR	U54	NT
HD16	NR	NR	U54
HD17	NR	NR	NR
HD18	NR	U90	NR
HD19	NR	U90	NR
HD20	U90	U54 and U90	NT
HD21	NR	NR	NR
HD22	NR	NR	NT
HD23	NR	NR	NT
HD24	NR	U11	NT
HD25	NR	NR	NT
HD26	U54	NR	NT
HD27	NR	NR	NT
HD28	NR	U39	NT
HD29	NR	U54 and U90	NT
HD30	U90	U11, U54 and U90	NT
Total	13/30	20/30	5/11

*NR = No response. ** NT = Not tested.

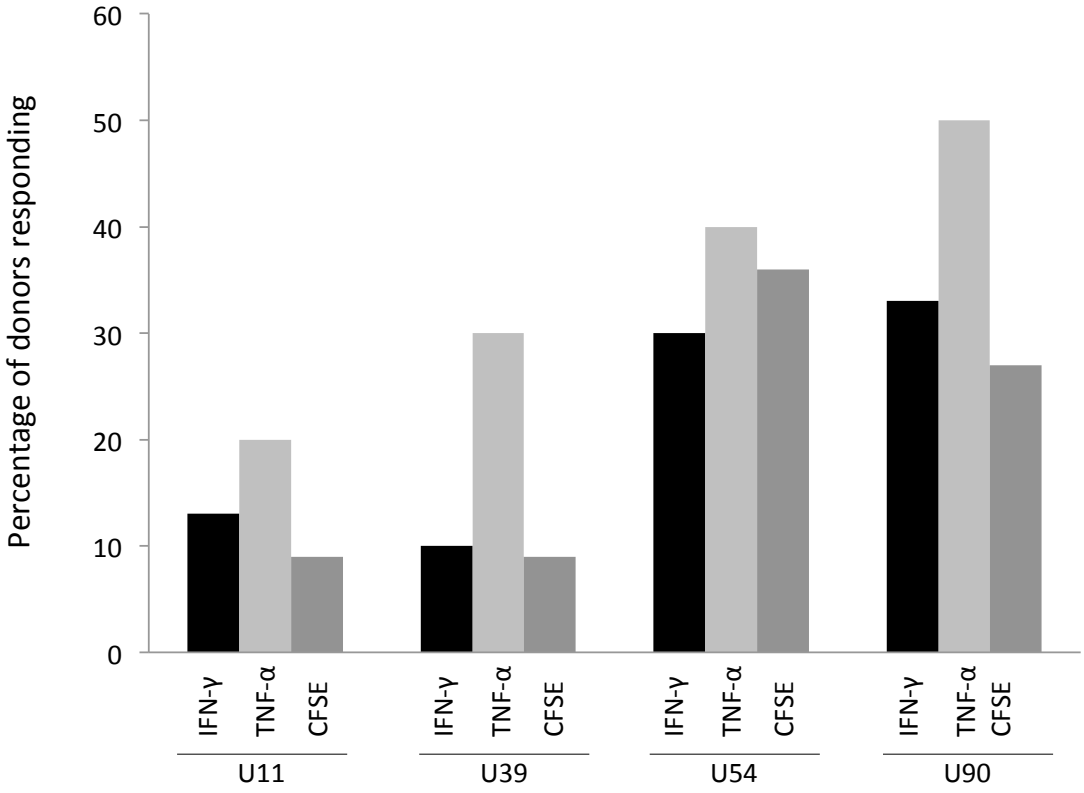


Figure 3.9 Percentage of donors responding to HHV6B antigens U11, U39, U54 and U90 analysed *ex-vivo* by intracellular cytokine staining for CD8+ IFN-γ+ or TNF-α+ cells and by CFSE proliferation assay

A total of 30 donors were analysed *ex-vivo* by ICS for HHV6B antigens U11, U39, U54 and U90 and 11 donors analysed *ex-vivo* by CFSE proliferation assay. The chart illustrates the percentage of donors responding to each HHV6B antigen tested for CD8+ IFN-γ+, TNF-α+ or reduced CFSE staining.

3.2 Summary

These studies to detect HHV6B T cell responses *ex-vivo* revealed that, in comparison to HCMV, responses were in general very weak or undetectable. Using ICS (for both IFN-γ and TNF-α) and CSFE proliferation assays values for HHV6B-specific T cells in PBMC ranged from 0.02-0.2%. CD8+ T cell responses were detected against all the antigens tested, with U54 and U90 being the most immunogenic.

Chapter 4

4. *In-vitro* expansion and analysis of T cell responses to HHV6B antigens U11, U39, U54 and U90 in a panel of healthy donors

Attempts to characterise CD8+ T cell responses specific for HHV6B antigens U11, U39, U54 and U90 directly *ex-vivo* using both ICS and CSFE staining showed that responses were present, but that they were at a very low frequency (less than 0.2% of CD8 T cells). Therefore, to maximise the ability to detect HHV6B antigen-specific T cells and obtain a better understanding of the immunogenicity of the 4 antigens being studied, a short-term *in-vitro* reactivation protocol was utilised to generate a polyclonal culture enriched with HHV6B-specific T cells. The experimental approach is outlined in Figure 4.1. PBMC were stimulated with pepmixes representing either U11, U39, U54 and U90, and cultures were maintained for 10 days at 37°C, with IL2 being added to cultures from day 3. Polyclonal cultures were then analysed in two ways. Firstly, T cell responses to individual HHV6B antigens were analysed by IFN-γ ELISpot. Secondly, the expanded HHV6B-specific T cell culture were characterised for the percentage of CD4+ and CD8+ T cells by flow cytometry.

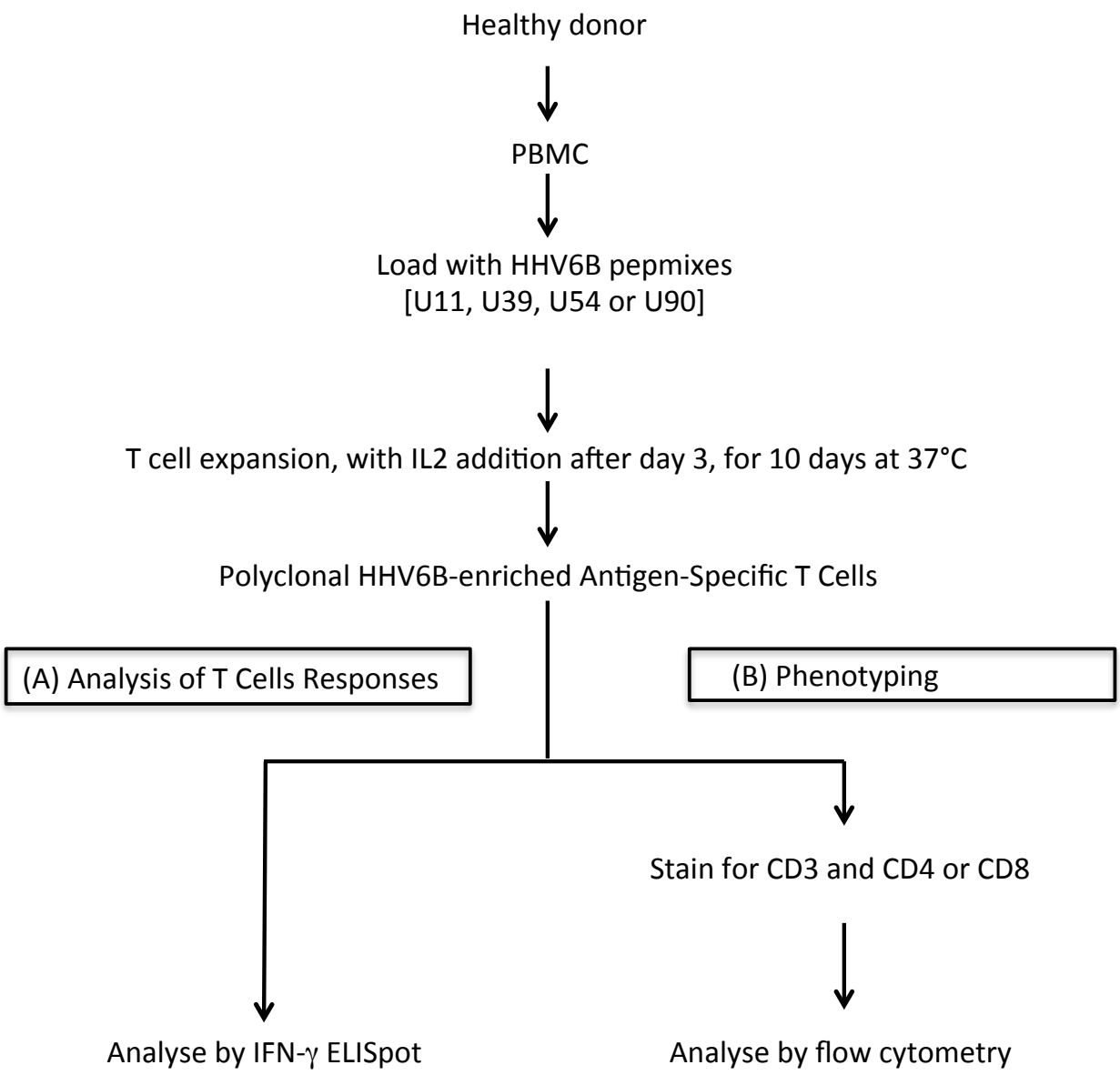


Figure 4.1 Flowchart showing the approach used for short-term *in-vitro* reactivation of HHV6B-specific T cells.

PBMC were stimulated with pepmixes representing HHV6B U11, U39, U54 or U90, and cultured for 10 days at 37°C, with addition of IL2 after day 3. After 10 days the polyclonal HHV6B enriched antigen-specific T cells were (A) analysed for HHV6B T cells responses by IFN-γ ELISpot after simulation with the individual U11, U39, U54 or U90 pepmixes, or (B) or anaylsed by flow cytometry to determine the percentage of CD4 + or CD8+ T cell in the polyclonal culture.

4.1 IFN-γ ELISpot analysis of T cell responses after short-term *in-vitro* stimulation of donor PBMC with individual antigen pepmixes

The work in this section has focused on utilising a short-term *in-vitro* stimulation step to attempt to enhance detection of HHV6B T cell responses. After stimulation of PBMC for 10 days with individual pepmixes, polyclonal cultures were then stimulated overnight with the same individual pepmixes before being analysed by IFN-γ ELISpot. Throughout this chapter data is presented as IFN-γ spot forming cells (SFC) per 10^5 cells. In the ELISpot ConA stimulated PBMC was used as a positive control throughout, and as a negative control cells were stimulated with an irrelevant pepmix corresponding to the VP1 antigen from BK virus. A positive ELISpot result was considered as a value equal to or greater than 10 SFCs per 10^5 cells above the negative control value (Boritz *et al.*, 2004). Before using this method to investigate HHV6B responses, to validate the approach similar experiments were undertaken to analyse responses to known HCMV immunogenic antigens.

4.1.1 Analysis of HCMV-specific T cell responses by ELISpot after 10-day *in-vitro* stimulation.

These validation experiments were carried out using both sero-positive and sero–negative HCMV donors, and pepmixes representing four immunogenenic HCMV antigens, namely IE1, IE2, pp65 and gB (Khan, 2007). The first donor analysed was HD15, an HCMV sero-positive donor with an HLA type A1, A2, B7, and B44, so would be expected to have T cells specific to the pp65 HLA-A2 restricted peptide NLVPMVATV (NLV, aa495-503) and pp65 HLA-B7 restricted peptide TPRVTGGGAM (TPR, aa417-426) (Wills *et al.*, 1996a). The base line level of T cells specific for NLV

and TPR peptides were measured by IFN-γ ELISpot. HD15 had a low, but detectable response to NLV and TPR of 4 and 8 SFC/10^5 PBMC, respectively (Figure 4.2A). PBMC from this donor where then stimulated with pp65 pepmix for 10 days, with addition of IL2 from day 3. The expanded pool of polyclonal T cells were then assayed by IFN-γ ELISpot, using the NLV and TPR peptides as the stimulatory. There was a significant enrichment of NLV and TPR -specific T cells to 120 and 147 SFCs/10^5 T cells, respectively and DMSO gave 62 SFCs/10^5 T cells (Figure 4.2B).

This donor was also shown to have a weak but detectable response to IE2 of 0.02% of both CD4 and CD8 T cells, as measured previously using IFN-γ ICS. The base line levels of T cells specific for HCMV IE2 were measured by IFN-γ ELISpot, using an IE2 pepmix as the stimulatory peptides. The results in Figure 4.3A, show that HD15 has a low, but detectable response to IE2 of 13 SFC/10^5 PBMC, generally corresponding to the levels of IE2-specific T cells measured previously using IFN-γ ICS (0.02%). PBMC from this donor where then stimulated with IE2 pepmix for 10 days, with addition of IL2 from day 3. The expand pool of polyclonal T cells were then assayed by IFN-γ ELISpot, using the IE2 pepmix as the stimulatory peptides. There was a significant enrichment of IE2-specific T cells to 400 SFCs/10^5 input cells, which after subtraction of the DMSO negative controls reflected a 26-fold expansion in IE2-specific IFNγ-positive T cells (Figure 4.3B).

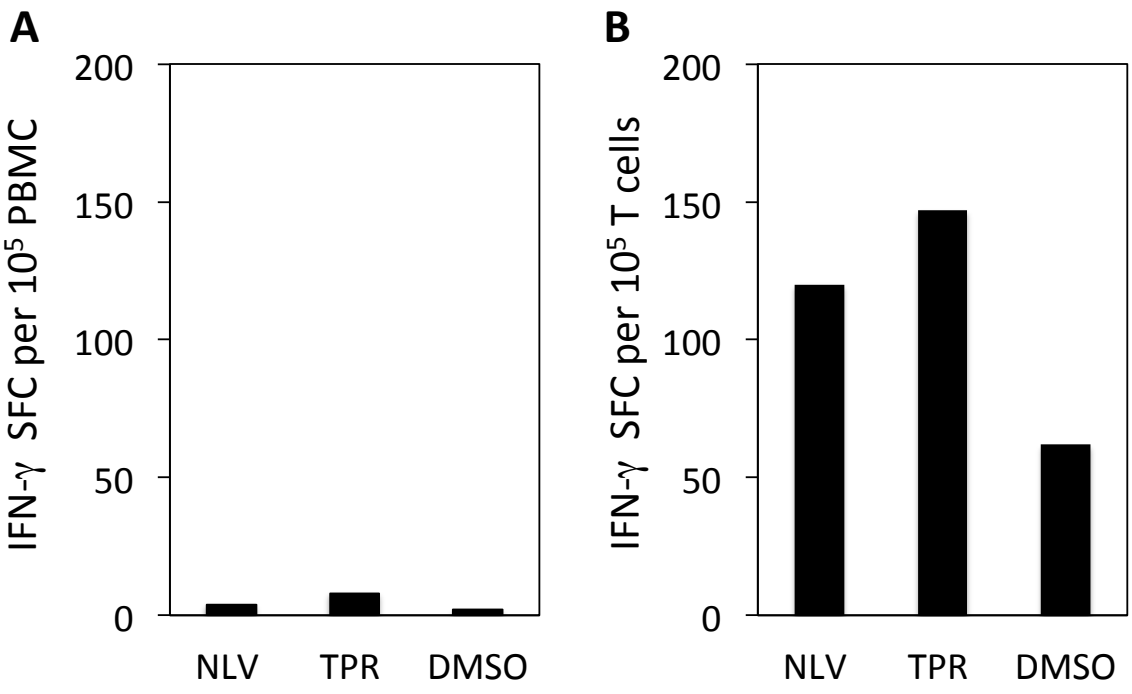


Figure 4.2 An *in-vitro* stimulation step can successfully expanded HCMV-pp65 specific T cells in CMV positive donor HD15

(A) The chart illustrates the base level of T cell responses, PBMC were isolated from donor HD15 and $1x10^5$ were stimulated with HCMV-NLV and -TPR peptides for 16 hours, before being analysed for production of IFN-γ by ELISpot (B) PBMC from the same donor were cultured for 10 days in the presence of HCMV-pp65 antigen and T cells expression of IFN-γ were determined by ELISpot using the NLV and TPR peptides as the stimulatory.

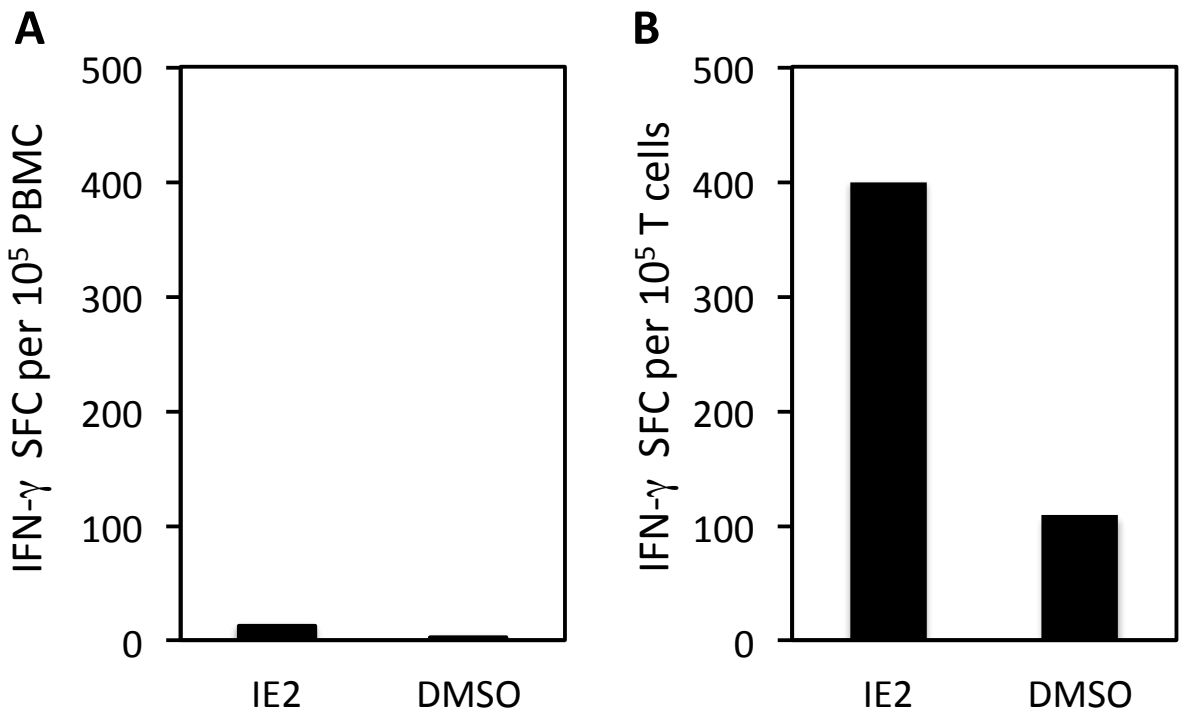


Figure 4.3 An *in-vitro* stimulation step can successfully expanded HCMV-IE2 specific T cells in HCMV sero-positive donor HD15

(A) The base line levels of T cell responses to IE2 were measured by direct analysis of PBMC via IFN-γ ELISpot using HCMV IE2 whole antigen pepmix. (B) PBMC from the same donor were than cultured for 10 days in the presence of HCMV-IE2 antigen, and IL2 from day 3, the ELISpot repeated using this HCMV IE2 enriched T cell population. PBMC and the enriched cell population were pulsed with DMSO as the negative control in the ELISpot assay.

To ensure that this approach was specific for reactivation of memory HCMV-specific T cells, these experiments were repeated using the four HCMV antigen pepmixes (IE1, IE2, pp65 and gB) and four HCMV sero-negative donors, along with a further two sero-positive donors. After stimulation of PBMC for 10 days with HCMV IE1, IE2, pp65 and gB pepmixes, polyclonal cultures were then assayed by IFN-γ ELISpot for antigen-specific T cells, using the pepmixes as stimulus. For both sero-positive donors (HD24 & HD32), there were significant levels of HCMV-specific T cells detected against all four antigens. For donor HD24 responses ranged from 54 SFCs per 10^5 cells for IE2, to 62 SFCs per 10^5 cells for pp65, to 68 SFCs per 10^5 cells for gB and 330 SFCs per 10^5 cells for IE1. HD32 had a detectable responses as follows: IE1 58 SFCs per 10^5 cells, IE2 246 SFCs per 10^5 cells, pp65 244 SFCs per 10^5 cells, and gB 46 SFCs per 10^5 cells (Figure 4.4). The base line level of HCMV antigen-specific T cells was not measured by ELISpot for these donors. Data was available from ICS staining (see Appendix Table 8.2), which indicated that donor HD24 had detectable responses to IE1 (0.31%) and pp65 (1.92%). Whereas, donor HD32 had detectable responses to pp65 (0.28%) and IE2 (0.57%). Thus, again there was significant expansion of HCMV-specific T cells in these donors after the short-term stimulation with individual pepmixes. A total of 4 HCMV sero-negative donors were screened, and representative examples from donors HD18 and HD56 are shown in Figure 4.4. Importantly, no HCMV-specific T cells were detectable in any of the sero-negative donors tested (Table 4.1). Thus, this approach was thought to be suitable to expand T cells specific for HHV6B, and that this would represent memory response rather than primary responses.

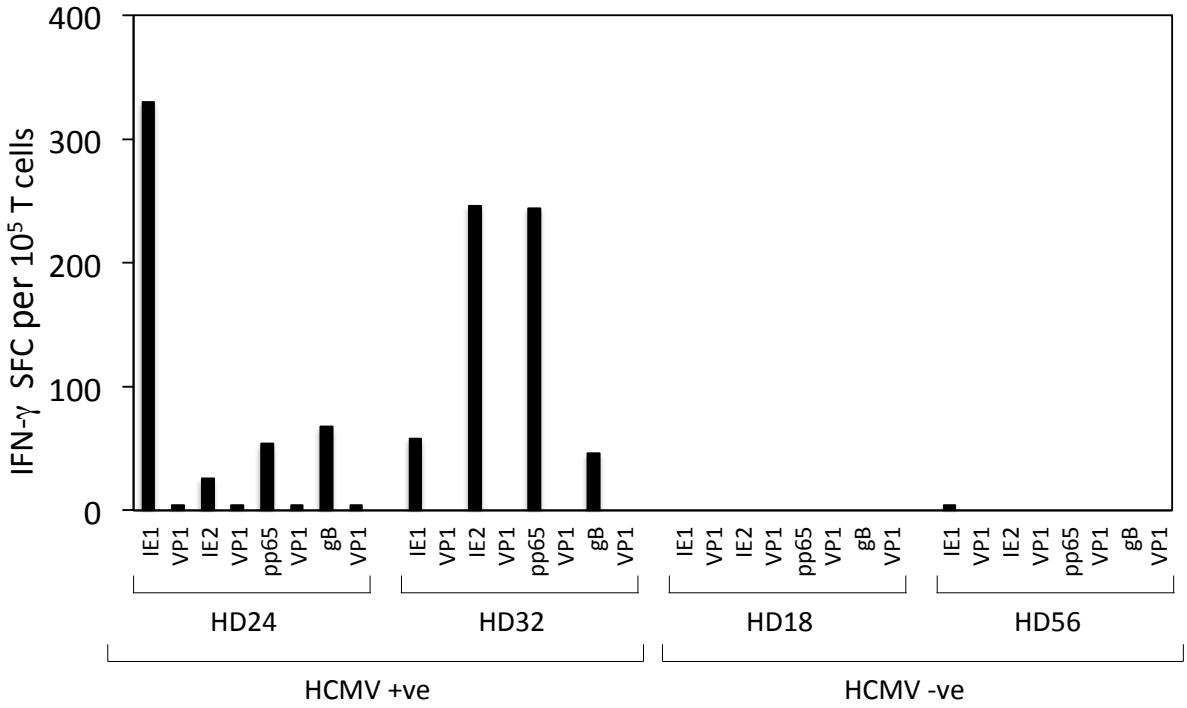


Figure 4.4 ELISpot analysis of the frequency of T cell response to HCMV antigens in HCMV-sero-positive and sero-negative donors after a short term *in-vitro* reactivation

PBMC were cultured for 10 days in the presence of either HCMV IE1, IE2, pp65 and gB pepmixes, with IL2 added from day 3, and the frequency of antigen specific T cells expressing IFN-γ were determined by ELISpot. Donors HD24 and HD32 are HCMV sero-positive, whereas donors HD18 and HD56 are HCMV sero-negative. Cells stimulated with an irrelevant pepmix corresponding to the VP1 antigen from BK virus served as a negative control in ELISpots.

Table 4.1 Summary of T cell responses to HCMV antigens IE1, IE2, pp65 and gB in 8 donors analysed *in-vitro* by ELIspot for IFN-γ

Donor	CMV Status	IE1	IE2	pp65	gB
HD10	-	-	NT	-	NT
HD15	+	+	+	+	NT
HD18	-	-	-	-	-
HD24	+	+	+	+	+
HD32	+	+	+	+	+
HD44	+	+	+	+	+
HD53	-	-	-	-	-
HD56	-	-	-	-	-

NT= not tested

4.1.2 Analysis of the breadth of T cell responses to HHV6B antigens U11, U39, U54 and U90 in healthy carriers

In the previous section, a short *in-vitro* stimulation step was shown to expand HCMV-specific T cell responses in HCMV sero-positive but not sero-negative donors. Thus, this approach was now utilised to attempt to expand HHV6B-specific T cell responses, with the overall aim of determining the breadth of T cell responses to the four HHV6B antigens U11, U39, U54 and U90. PBMC were stimulated *in-vitro* with individual pepmixes for 10 days, with IL2 being added after day 3. The frequency of HHV6 antigen-specific T cells in the expanded polyclonal T cell populations was then measured by IFNγ ELISpot. The first donor analysed was HD27. PBMC from HD27 were stimulated with HHV6B U11, U39, U54 and U90 pepmixes cultured for 10 days and the number of expanded T cells specific for the stimulatory pepmix determined by IFN-γ ELISpot (Figure 4.5). IFNγ+ve T cells were detected against all four HHV6B antigens, at frequencies ranging from 94 SFCs per 10^5 cells for U54 and 104 SFCs per 10^5 cells for U39, up to 260 SFCs per 10^5 cells for U11 and 286 SFCs per 10^5 cells for U90. Responses detected against the irrelevant VP1 pepmix were low, less than 5 SFCs per 10^5 cells. Based on the *ex-vivo* analysis of PBMC chapter 3, this donor was not predicted to have any responses against HHV6B antigens tested (see earlier, Table 3.5) Thus, the additional step of a 10-day *in-vitro* expansion provided a more sensitive approach for determining the immunogenicity of the individual HHV6B antigens.

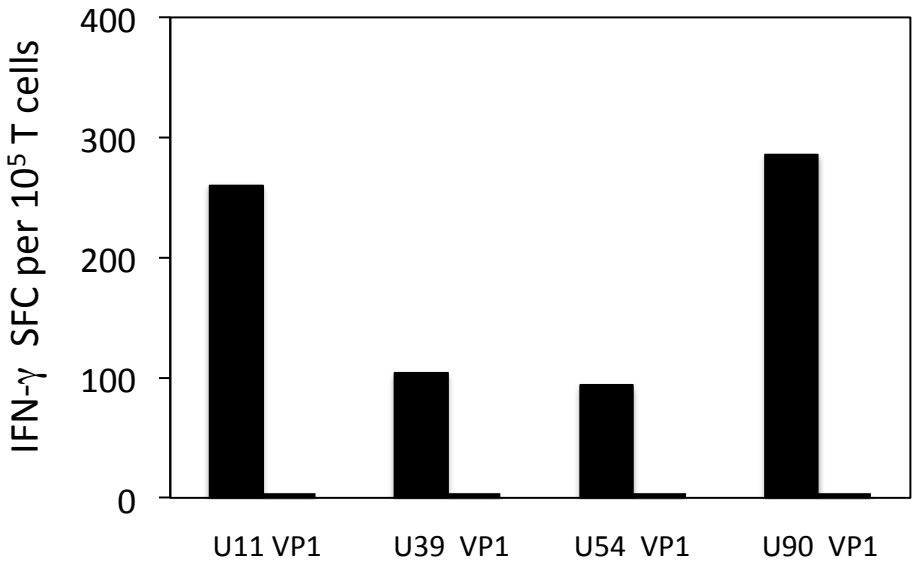


Figure 4.5 ELISpot analysis of the frequency of T cell response to HHV6B antigens U11, U39, U54 and U90 in donor HD27 after a short term *in-vitro* reactivation

PBMC from donor HD27 were stimulated with HHV6B pepmixes U11, U39, U54 and U90, and cultured for 10 days, in the presence of IL2 after day 3. The frequencies of HHV6B -specific T cell were then measured by IFN-γ ELISpot. Results are presented as spot-forming cells (SCF) per 10^5 T cells. The irrelevant pepmix, VP1, was used as a negative control in the ELISpot assays.

Data from three further donors, HD05, HD30 and HD07, analysed by ELISpot after a short-term stimulation with each of the HHV6B pepmixes are shown in Figure 4.6. HD05 had a detectable T cell response against all HHV6B antigens tested, with levels of IFNγ-postive antigen-specifc T cells as follows: U11 346 SFCs per 10^5 cells, U39 62 SFCs per 10^5 cells, U54 372 SFCs per 10^5 cells, and U90 272 SFCs per 10^5 cells. HD30 also had a detectable T cell response against all HHV6B antigens tested, although generally lower than the two previous donors. Responses detected were U11 98 SFCs per 10^5 cells, U39 90 SFCs per 10^5 cells, U54 72 SFCs per 10^5 cells, and U90 96 SFCs per 10^5 cells, whereas, HD07 had a detectable T cell response against only two of the HHV6B antigens, U90 (360 SFCs per 10^5 cells) and U11 (98 SFCs per 10^5 cells). The responses from elispots assays with these 3 donors were very high compare with earlier data from intracellular staining and CFSE assays.

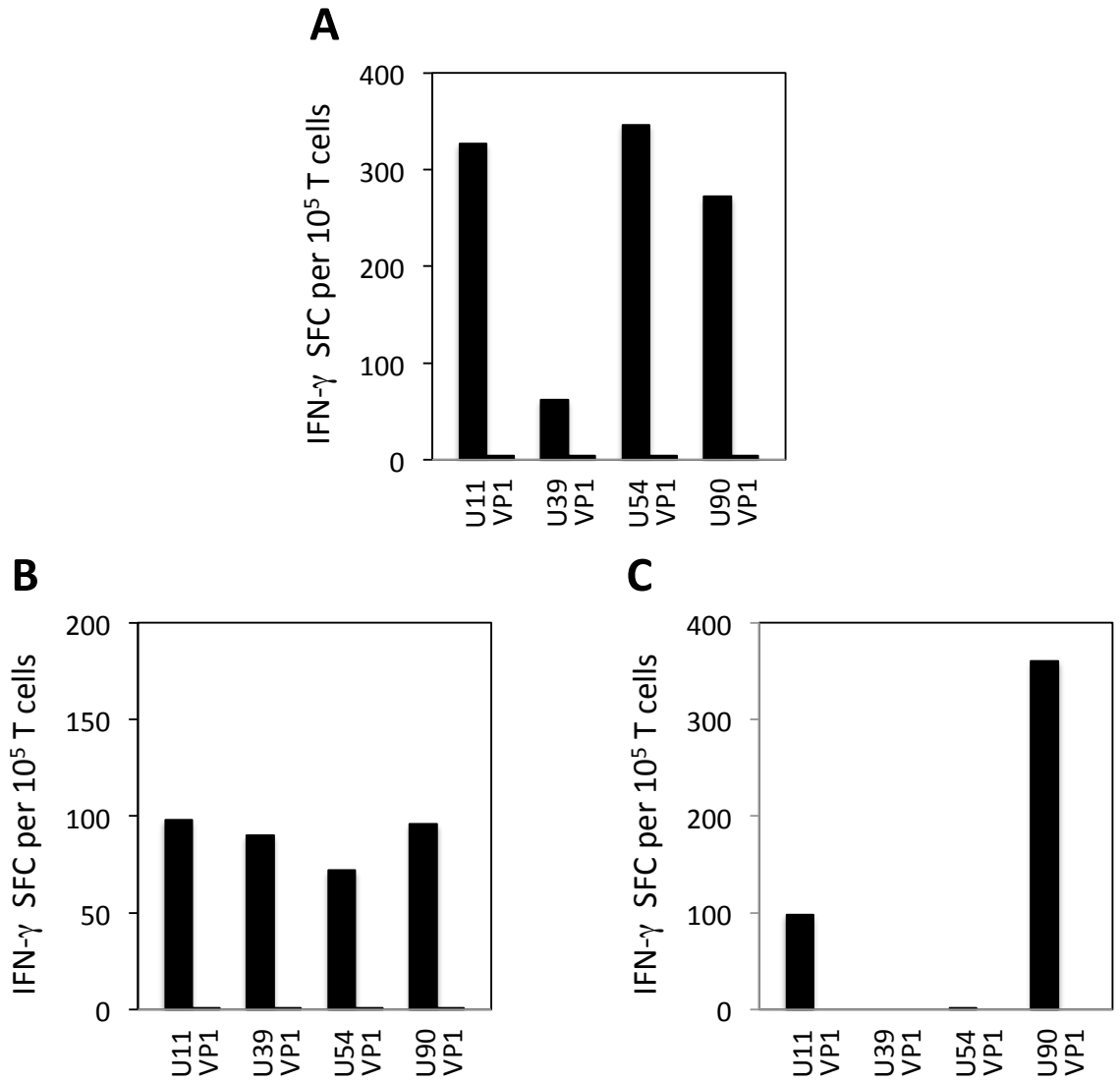


Figure 4.6 Identification of T cell response to U11, U39, U54 and U90 after short term *in-vitro* reactivation

PBMC from donors HD05 (A), HD30 (B) and HD07 (C) were stimulated with HHV6B pepmix U11, U39, U54 and U90, and cultured for 10 days in the presence of IL2. HHV6B -specific polyclonal T cells were then screened by IFN-γ ELISpot. Results are presented as spot-forming cells (SCF) per 10^5 T cells. Pepmix representing BK-VP1 was used as negative control.

A total of 25 donors were analysed in this way. The overall results are shown in Table 4.2, which shows that all donors tested responded to at least one HHV6B antigen, with 15 donors responding to all four antigens, 2 donors responding to three antigens, 6 donors responding to two antigens and finally 2 donors responding to only one antigen (Figure 4.7). The individual magnitudes and mean frequencies of the responses against each antigen are shown in Figure 4.8. The mean frequency of T cells responding to U11 was 93 SFC per 10^5 of total T cells, with the range being from 14 to 372 SFC per 10^5. The mean frequency of T cells responding to U39 was 74 SFC per 10^5 of total T cells, with a range of 10 to 186 SFC per 10^5). The mean frequency of T cells responding to U54 was 121 SFC per 10^5 of total T cells, with a range of 32 to 346 SFC per 10^5). Finally, the mean frequency of T cells responding to U90 was 115 SFC per 10^5 of total T cells, with a range from 10 to 376 SFC per 10^5.

Table 4.2 Summary of T cell responses to HHV6B antigens U11, U39, U54 and U90 in 25 donors detected by IFN-γ ELIspot after a short-term *in-vitro* reactivation

Donor	U11	U39	U54	U90
HD02	244	186	32	252
HD05	372	62	346	272
HD07	98	0	2	360
HD10	136	120	149	93
HD11	140	188	182	192
HD18	3	77	125	144
HD24	26	28	95	15
HD27	260	104	94	286
HD30	98	90	72	96
HD31	254	162	254	376
HD32	14	4	150	0
HD44	108	38	90	10
HD45	128	130	196	134
HD46	0	172	134	146
HD47	148	92	228	166
HD48	16	102	52	152
HD49	40	116	118	44
HD50	20	0	122	2
HD51	4	2	160	0
HD52	4	4	64	0
HD53	0	0	80	10
HD54	40	10	84	10
HD55	0	50	0	60
HD57	86	120	39	44
HD58	90	0	6	154
Total out of 25	19	18	22	21

* The results are expressed as SFC per 10^5 cells after subtract the negative control from the individual reading. Numbers in the final row indicate numbers of positive donors.

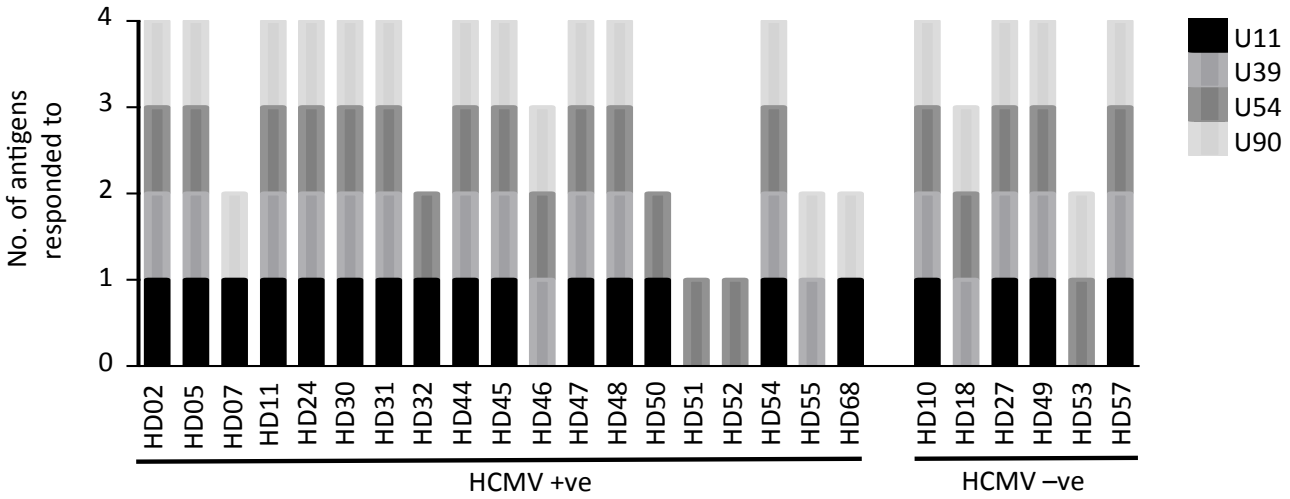


Figure 4.7 Summary data from 25 donors screened against HHV6B antigens U11, U39, U54 and U90

The overall results of all donors tested against the HHV6B antigen are shown.

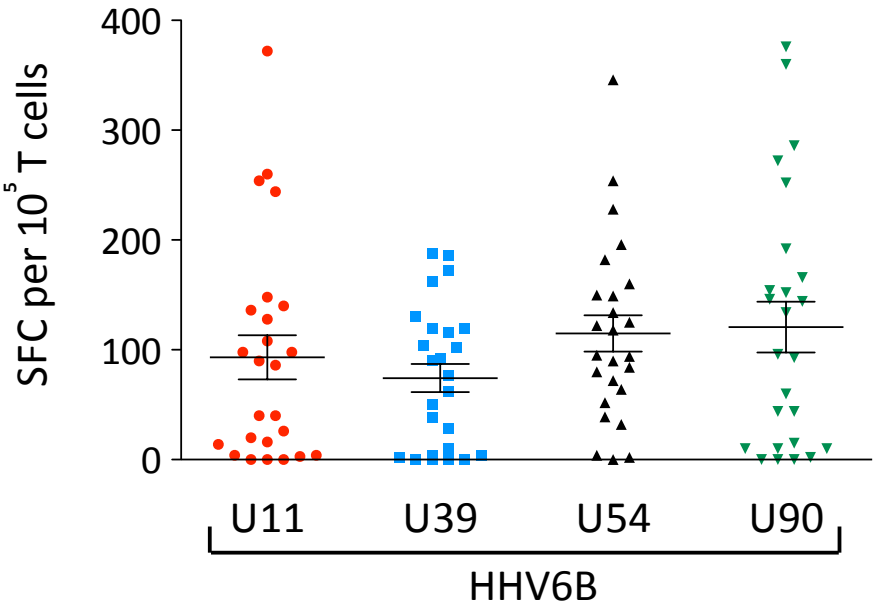


Figure 4.8 T cell responses to HHV6B antigens U11, U39, U54 and U90 in 25 donors analysed after *in-vitro* reactivation by IFN-γ ELISpot

The results are expressed as IFN-γ secreting T cells (SFC) against the HHV6B protein from 25 donors, the mean value ± SEM are shown.

Based on analysis of the mean frequency of SFC expanded by a 10-day culture, U54 was the most immunogenic, closely followed by U90, then U11 and U39. To determine the overall hierarchy of immune responses to the HHV6B antigens tested, the number of donors responding to each of the four antigens test was determined. Whilst all donors respond to a combination of the antigens (see earlier, Figure 4.8), HHV6B U54 was the most immunodominant with 22 out of 25 donors responding (88%), next was U90 with 21 out of 25 donors responding (84%), followed by U11 with 19 of the 25 donors responding (76%) and then U39 with 18 of the 25 donors responding (72%) (Figure 4.9). Thus, although all antigens were considered strongly immunogenic, the overall hierarchy of iummunodominance detect in this cohort of 25 donors showed that U54 was the most immunogenic followed closely by U90, then U11 and finally U39.

Of the 25 donors analysed 19 were HCMV sero-positive and 6 were sero-negative. Due to the level of similarity between the two β-herpesviruses, HHV6B and HCMV, there may be the potential of the HHV6B pepmixes to expand cross-reactive HCMV T cells. Thus, there was the possibility that data generated may not be truly reflective of the frequency of HHV6B-specific responses. When the level of responses against HHV6B U11, U39, U54 and U90 were compared between the HCMV sero-positive and sero-negative groups, the overall levels of responsive T cells were similar and the hierarchy was identical (Figure 4.10).

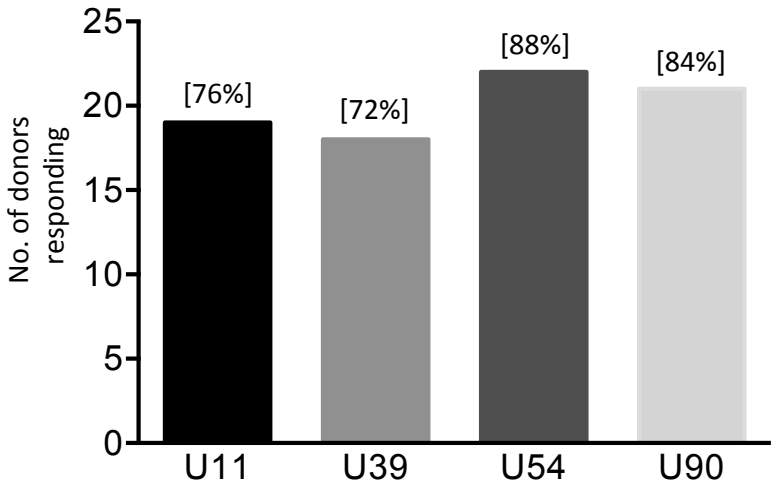


Figure 4.9 Percentage of donors eliciting T cell responses to HHV6B antigens U11, U39, U54 and U90 in 25 donors analysed after *in-vitro* reactivation by IFN-γ ELISpot

A total of 25 donors were analysed after *in-vitro* reactivation by IFN-γ ELISpot for HHV6B antigens U11, U39, U54 and U90. The highest number of donors (22/25) responded to U54, followed by U90 (21/25), U11 (19/25) and response to U39 was the lowest (18/25) among them.

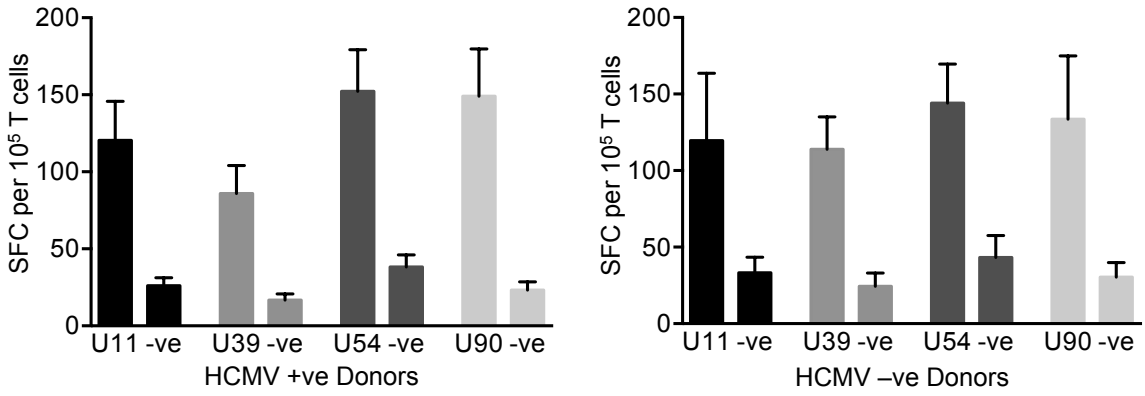


Figure 4.10 T cell responses to HHV6B antigens U11, U39, U54 and U90 in relation to CMV sero-positivity.

Figure showing similar pattern and magnitude of T cell responses in CMV positive (left) and CMV negative donors (right). For each antigen, the negative control (DMSO) is shown to the right of the pepmix.

4.2 Determination of the frequency of CD4+ and CD8+ T cells in the expanded HHV6B-specific polyclonal T populations

The previous section has shown that 100% of donors tested had T cell responses directed against HHV6B that could be expanded *in-vitro* by short-term reactivation with antigen pepmixes. Further, all of the antigens tested were able to stimulate expansion of HHV6B-specific T cells. However, this analysis was carried out at the level of total T cells, and gave no indication of the CD4+ and CD8+ T cell composition of these expanded cultures and subsequently what type of HHV6B-specific T cell was being expanded. Thus, the frequencies of CD4+ and CD8+ T cells in 10-day polyclonal cultures, stimulated with each HHV6B pepmix, were measured by flow cytometry.

An example of this analysis for donor HD49 is shown in Figure 4.11. After a 10-day expansion with each antigen pepmix, cells were stained with CD3 and either CD4 or CD8, and the frequencies of CD3+CD4+ and CD3+CD8+ cells determined. As seen in figure 4.10, the frequency of CD3+CD4+ cells present in the respective pepmix-expanded cultures were 64.5% (U11), 70.4% (U39), 61.8% (U54) and 68.3% (U90). The corresponding levels of CD3+CD8+ cells were 27% (U11), 22.7% (U39), 28.7% (U54) and 25.4% (U90). Thus, for this donor the vast majority of expanded T cells for each HHV6B antigen were CD4+.

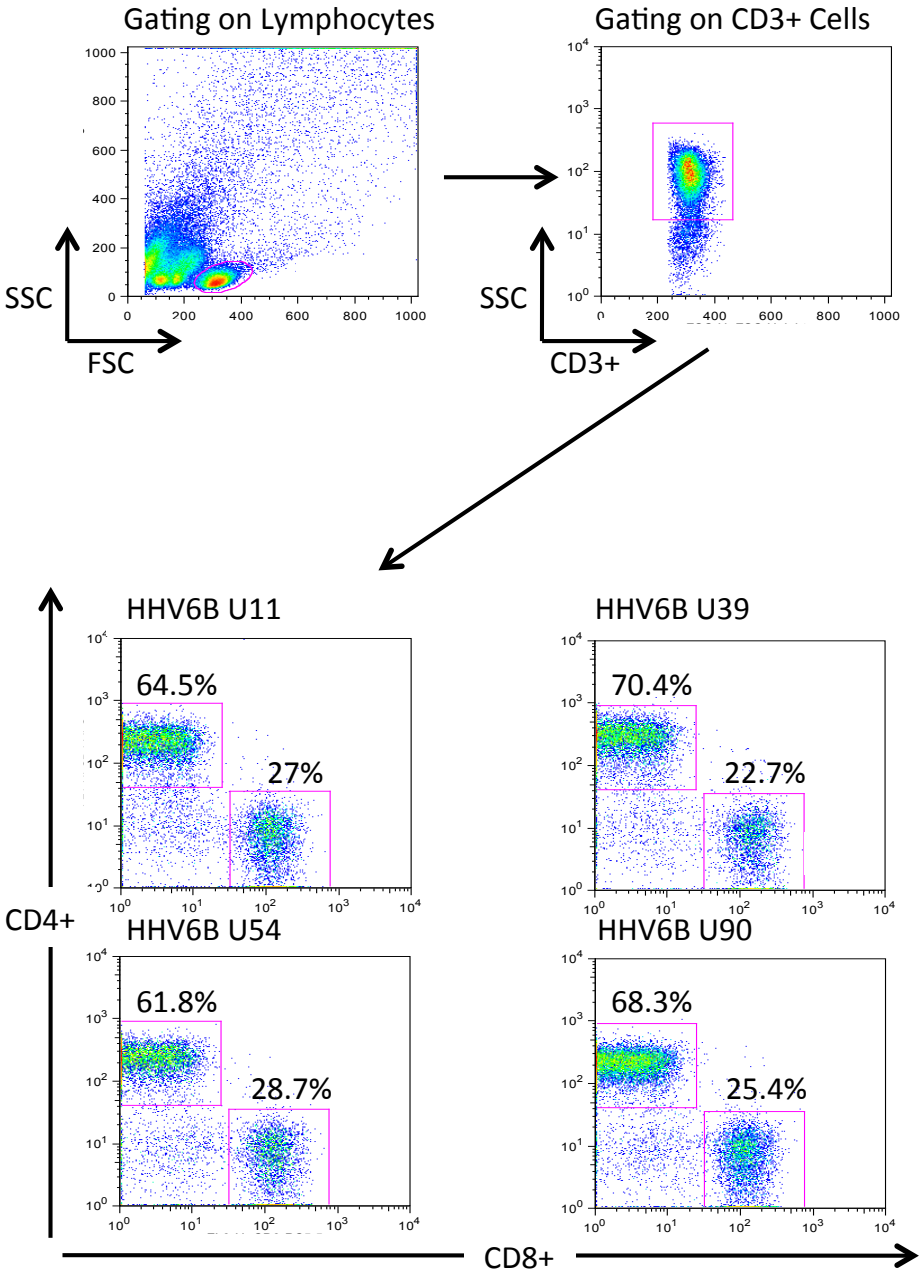


Figure 4.11 Phenotypic analysis of polyclonal culture of HHV6B-specific T cells in donor HD49 after a short-term *in-vitro* reactivation

PBMC were isolated from donor HD49 and $5x10^5$ were stimulated with HHV6B whole antigen pepmixes for either U11, U39, U54 orU90 for 10 days. Lymphocytes were identified based on their forward- and side-scatter properties. Subsequently, CD3 was used to identify T cells among the previously selected lymphocytes. CD4+ T cells and CD8+ T cells were identified for polyclonal culture of HHV6B-enriched antigen-specific T cells. Values indicated denote the percentage of CD4+ or CD8+ cells. Data was collected on a BD FACSCalibur and analysed using FlowJo software (Treestar).

A total of 10 donors were analysed, and the percentage values for CD3+CD4+ and CD3+CD8+ cells are shown in Table 4.3. Overall, the pattern observed was similar to that for donor HD49. However, interestingly donors HD32, HD50 and HD51 were found to have significantly higher levels of CD3+CD8+ cells (42%, 41% and 36% respectively) in U54 expanded T cell populations. As shown in the previous chapter, U54 was deemed to be immunogenic for CD8+ responses, although these donors were not included in that part of the study. A summary of the results is shown in Figure 4.12. The mean frequency of CD3+CD4+ and CD3+CD8+ T cells for each antigen pepmix is shown in Figure 4.12. Overall, the values for each antigen are similar, with only a slight increase in the mean value of CD8+ cells for U54 over the other antigens, reflecting the values highlighted above. The mean frequency detected and range where as follows: CD3+CD4+ cells for U11 was 66.6% (range 42.8% to 77.3%); U39 was 67.9% (range 37.7% to 79.3%); U54 was 61.8% (range 42.6% to 80%); U90 was 67.4% (range 42.3% to 83.9%). CD3+CD8+ cells for in U11 was 21.3% (range.7% to 38.8%); U39 was 23.2% (range 0.2% to 27.6%); U54 was 28.7% (range 13.3% to 41.8%); U90 was 24.4% (range 1.3% to 27.1%).

Table 4.3 CD4 and CD8 phenotype and frequency of T cell lines after 10-day *in-vitro* stimulation with HHV6B antigens

Donor	CD3+CD4+ T cells				CD3+CD8+ T cells			
	U11	**U39**	**U54**	**U90**	**U11**	**U39**	**U54**	**U90**
HD02	77.1	79.3	80	83.9	13.9	14.2	13.3	11.6
HD05	66.3	74.2	73	NT	19.1	21.2	21.4	NT
HD07	66.8	68.2	68.1	63.6	23.5	25.5	24	26
HD27	NT	NT	NT	71.2	NT	NT	NT	20
HD31	68.7	66.1	64.2	68.5	23.9	24.9	25.2	23.3
HD32	52.4	66.5	45.2	NT	38.8	27.6	41.8	NT
HD48	69.6	67.6	58.1	63	16.7	23.7	31.1	27.1
HD49	64.5	70.4	61.8	68.3	27	22.7	28.7	25.4
HD50	NT	NT	46.9	66.5	NT	NT	40.8	26.1
HD51	42.8	37.7	42.6	42.3	0.713	0.21	36.2	1.26
Mean	58.4	62.8	61	68.6	19.3	24.5	29.8	31.2

NT = not tested

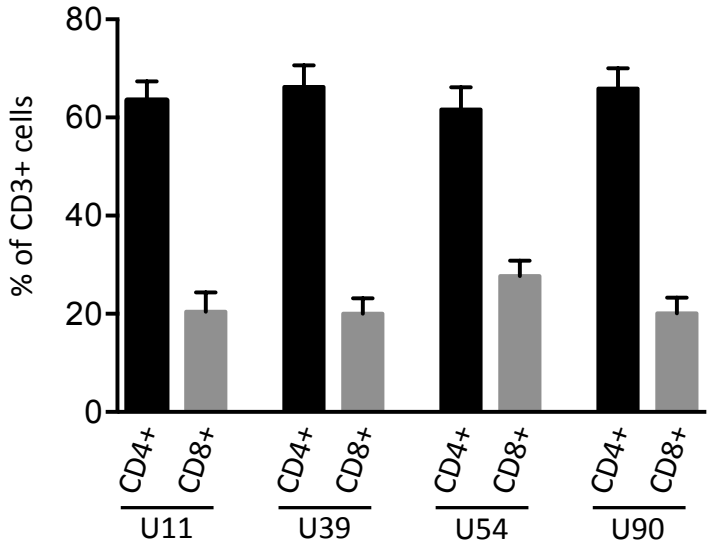


Figure 4.12 Phenotypic analysis of polyclonal cultures of HHV6B-specific T cells generated by short-term *in-vitro* reactivation with antigen pepmixes.

The mean percentage values, ± SEM, of CD3+CD4+ and CD3+CD8+ cells present in polyclonal culture of HHV6B-enriched antigen-specific T cells. The number of donors analysed for each antigen pepmix were as follows: U11 n=8, U39 n=8, U54 n=9 and U90 n=8.

4.3 Summary of the analysis of pepmix expanded HHV6B-specific T cell populations

In conclusion, despite the very low levels of HHV6B-specific T cells detected *ex-vivo* in chapter 1, an *in-vitro* stimulation step in the presence of IL-2 successfully expanded HHV6B antigen-specific T cells. T cell responses were detected against all HHV6B antigens tested (U11, U39, U54 and U90). All 25 donors analysed responded to at least one of the HHV6B antigen. A hierarchy of immunodominance was determined, with U54 being the most immunogenic, followed closely by U90 then U11 and U39. The expanded CD3+ T cell population consisted mainly of CD4+ T cells, with a significantly lower percentage of CD8+ T cells.

Chapter 5

5. Identification and characterisation of HLA Class I restricted antigenic peptides in HHV6B U11 and U90

The previous chapter has shown that T cells responses to HHV6B antigens U11, U39, U54 and U90 can be successfully expanded *in-vitro*. The next stage was to characterise these responses in more detail, focusing specifically of HLA class I restricted CD8+ T cell responses, which will provide valuable information to support any future application of T cell therapy against HHV6B driven disease. This chapter will describe the mapping the minimal antigenic peptides, identifying the HLA restriction elements and determining whether such T cells were capable of killing HHV6B-infected cells. Although U54 was shown to be the most immunogenic of the four tested, there was very little real difference between this antigen and U90, with 84% of donors tested responding to U90. U11 was also recognised by the majority of donors (76%). Importantly, peptide libraries of individual 15-mer peptides, overlapping by 11aa, were available for both antigens (provided by Dr Ann Leen, Houston, USA). Thus, this chapter has focused on U11 and U90 and donors which were shown to have CD8+ T cell responses to these antigens that could be expanded *in-vitro*.

Briefly, using the same approach as in Chapter 4 PBMC from selected donors were reactivated *in-vitro* with U11 or U90 pepmixes. These U11 or U90-specific expanded polyclonal T cell populations were then characterised in more detail. Initially, to attempt to identify novel CD8+ T cell epitopes in these antigens expanded polyclonal cultures were screened by ELISpot using the 15-mer peptide libraries. These libraries consisted of 267 15-mers for U90 and 212 15-mers for U11. To maximise the use of T cell cultures, these peptide libraries were arranged into mini-pools such that each

peptide was present in 2 pools. This generated 33 mini-pools for U90 and 30 mini-pools for U11. These peptide mini-pools were organised in a 2 dimension matrix that allowed for easy identification of individual target peptides, as described previously (Kern *et al.*, 2000) (see Figure 5.1). T cell lines were exposed to the relevant mini-pools, and positive responses were detected by IFN-γ ELISpot. These responses, which could be mapped to individual 15-mers, were then analysed in more detail using shorter peptides to map minimal epitopes. HLA restricting elements were mapped using autologous and partially matched target cells. Finally, The T cell lines were tested functionally for their ability to recognising HHV6B-infected target cells. An outline of the approaches used is shown in Figure 5.2.

A

U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
20	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
21	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
22	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85
23	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
24	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
25	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136
26	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
27	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
28	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187
29	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204
30	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221
31	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

B

U11	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
17	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
18	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
19	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
20	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
21	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
22	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
23	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
24	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
25	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
26	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
27	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
28	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
29	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
30	209	210	211	212												

Figure 5.1 Representation of the HHV6-U90 and U11 mini-pools of individual 15-mer peptides

The two dimensional grids show the mini-pools of individual 15-mer peptides for U90 (A) and U11 (B). For the 267 15-mer peptides representing U90, there are 33 mini-pools containing either 15, 16 or 17 peptides. Each U90 15-mer is present in two pools. For the 212 15-mer peptides representing U11, there are 30 mini-pools containing either 14, 15 or 16 peptides. Each U11 15-mer is present in two pools.

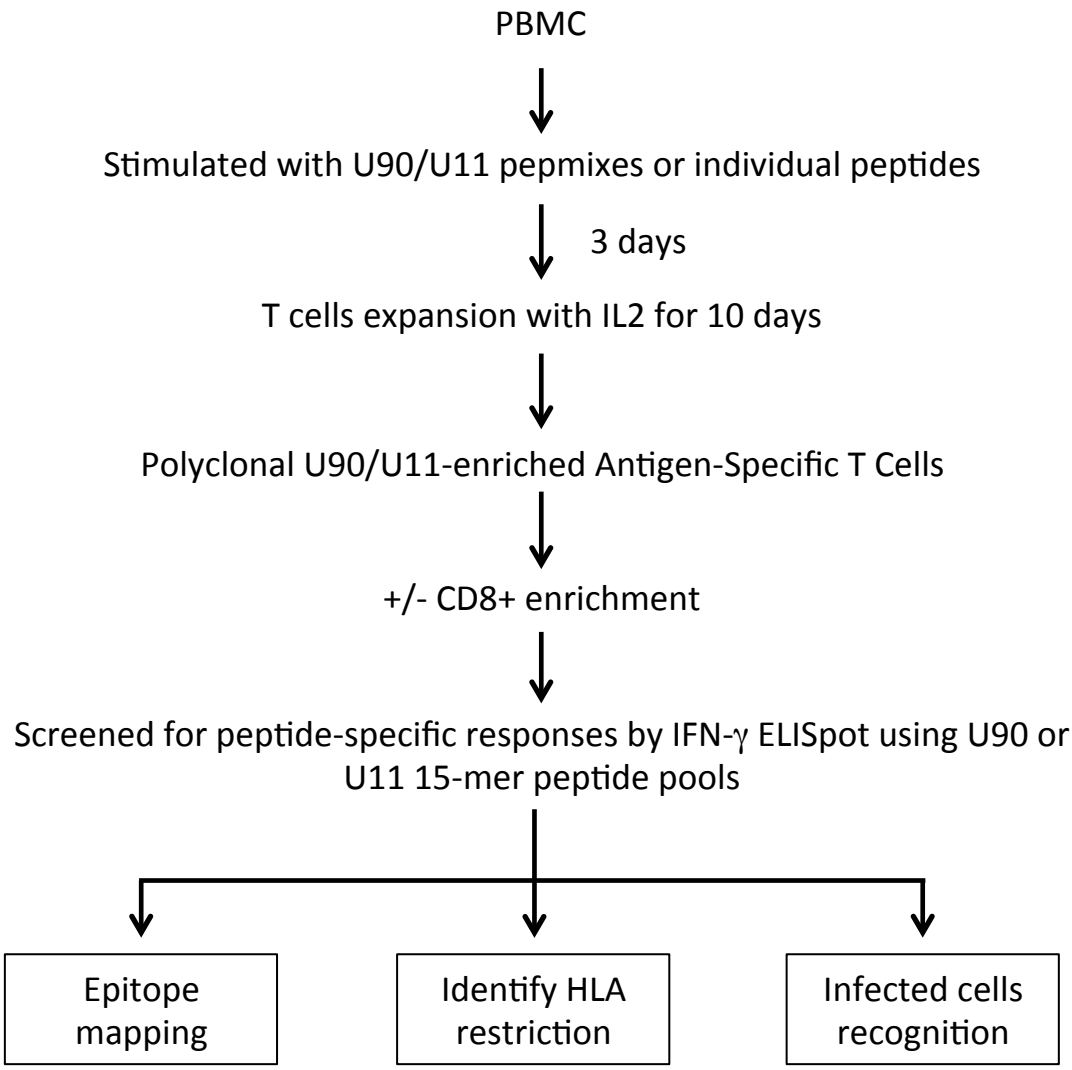


Figure 5.2 Overview of the process used to characterise CD8+ T cells responses to HHV6B U90 and U11

PBMC are stimulated with pepmixes representing HHV6B U90 or U11, or in some cases individual peptides were used, and cultures were maintained for 10 days at 37°C, with IL2 being added to cultures from day 3. Either total polyclonal T cell populations or populations enriched for CD8+ T cells, are screened by IFN-γ ELISpot using 15-mer mini-pools. Positive responses (T cell lines) are then analysed further by mapping the minimal peptide epitope, identification of the HLA restriction allele and investigating if they recognise HHV6B-infected cells.

5.1 Mapping of HLA Class I restricted T cell epitopes in HHV6B antigen U90

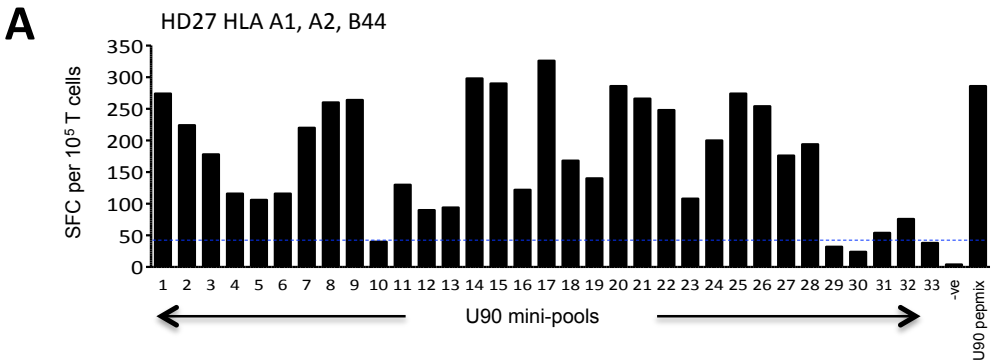
U90-specific T cells were identified in expanded T cell populations in 21 (out of 25) donors (see section 4.1.1). In this section, based on availability of donors, 6 were selected for further analysis in this chapter. These were donors HD27, HD53, HD57, HD49, HD10 and HD24. All but HD53 had been shown to responded to all four HHV6B antigens tested. HD53 was shown to respond to U90 and U54. PBMC were isolated, stimulated with U90 pepmixes in the presence of IL2 for 10 days, and subsequently analysed with the 15-mer mini-pools. In some cases, PBMC were subsequently stimulated with individual 15-mers to allow for follow up mapping experiments. For the first donor analysed (HD27), the initial ELISpot screen using the mini-pools was carried out with total T cell populations. However, using this approach the majority of pools gave a positive ELIspot results, likely to be due in part to CD4+ T cell responses (see Figure 5.3A). As the aim here was to identify and map CD8+ T cell epitopes, a CD4+ T cell depletion step was incorporated, to enrich for CD8+ T cells prior to screening by ELIspot with the 15-mer mini-pools.

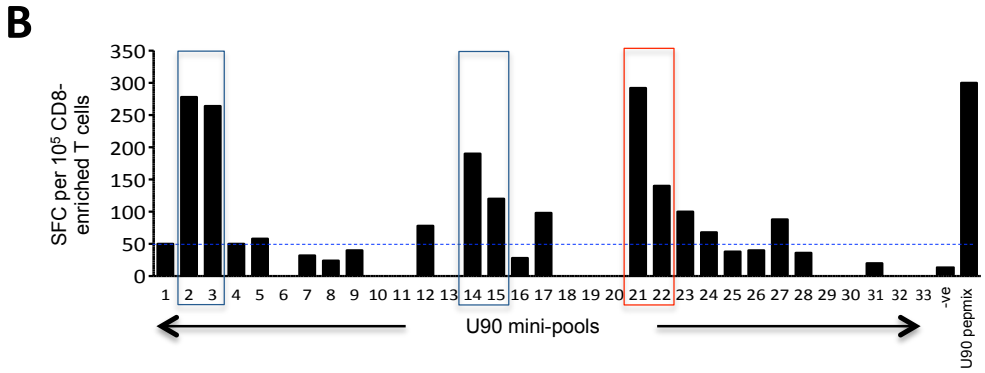
5.1.1 Mapping CD8+ T cell epitopes in donor HD27

Donor HD27 (HLA type HLA-A1, A2, B44) had strong responses to U90 of 286 SFCs per 10^5 cells after 10-day stimulation of PBMC with pepmix (Figure 4.5). To begin mapping CD8+ T cell responses, a pepmix-expanded total T cell population was incubated with the U90 mini-pools and analysed by ELISpot for IFN-γ secretion (Figure 5.3A). The U90 pepmix positive control gave a high level of SFC, and the pepmix representing the BK virus VP1 negative control was below 20 SFCs. As

mentioned above, when screening a total T cell population the majority of peptide mini-pools were above the cut off of 2-fold greater than the negative control. This was unmanageable in terms of further analysis, and as stated was likely in part to be due to the presence of CD4+ T cells responding to U90 peptides. Thus, CD4+ T cells were depleted leading to enrichment of CD8+ cells from 15% to 67% (Figure 5.3D). This CD8-enriched T cell population was then rescreened against the 15-mer mini pools giving a much cleaner result (Figure 5.3B). U90 mini-pools which were considered positive for HD27 were pools 2, 3, 12, 14, 15, 17, 21, 22, 23 and 27. Based on the strengths of the responses seen mini-pools 2, 3, 14, 15, 21 and 22 were selected for further analysis, and the overlap regions for these pools are highlighted in the 2 dimension matrix below the bar chart (Figure 5.3C). It is possible pools 12, 17, 23 and 27 also contain responses but these were not selected for further analysis. This highlighted individual 15-mer peptides 53 and 54 (overlap of 2/3 and 21), 65 and 66 (overlap of 2/3 and 22), 70 and 71 (overlap of 14/15 and 21), and 82 and 83 (overlap of 14/15 and 22). The CD8+ enriched T cell line was then screened by ELISpot against the individual 15-mers, including for thoroughness additional peptides on either side of the overlaps, for example for 15-mers 53 and 54 also peptides 52 and 55. Responses where only seen against overlapping individual 15-mers 53 and 54, and 82 and 83 (Figure 5.4), suggesting the other mini-pools hits were non-specific background. The 15-mer peptide 53 corresponds to aa209-223 (ASESRDLLMDLKANM) and 54 corresponds to aa213-237 (RDLLMDLKANMNNQF). The 15-mer peptide 82 corresponds to aa325-339 (IHINCKNLITAAKNI) and 83 corresponds to aa329-343 (CKNLITAAKNIGIAV). The overlap of peptides 82 and 82 includes the sequences NLITAAKNI (aa331-339) and the sequence ITAAKNIGI (aa333-

341) (Figure 5.5). The 9-mer sequences had previously been identified as potential HLA-A2 binding peptides using SYFPEITHI epitope prediction programme (N. Khan, personal communication). Importantly, this donor was HLA-A2 positive. Thus, as PBMC and expanded T cells populations were limiting from this donor, the next experiments focused on investigating if responses mapped to these 9-mers. The enriched T cells were screened by ELISpot against the 15-mer peptide 85 CKNLITAAKNIGIAV and both putative HLA-A2 restricted 9-mers. Both NLITAAKNI and ITAAKNIGI peptides were recognised in the ELISpot assay (Figure 5.5), suggesting that these are indeed HLA A2-restricted T cells epitopes from HHV6B U90, termed NLI and ITA (based on the first three amino acids). However, no addition fine mapping was carried out. Further analysis of these epitopes (HLA mapping and presentation in virus-infected cells) is presented in section 5.3 and 5.4 (this is the case for all epitopes identified and present in section 5.2). The potential response to the overlapping 15-mer peptides ASESRDLLMDLKANM and RDLLMDLKANMNNQF was not followed up but may represent an additional U90 T cell epitope recognised by this donor.





C

U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
20	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
21	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
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27	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
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31	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

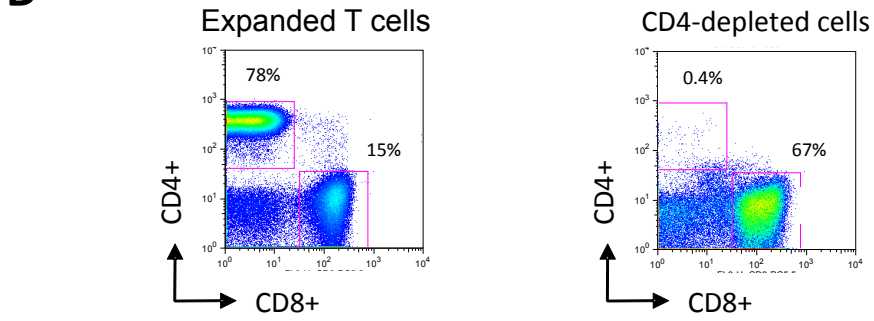


Figure 5.3 Screening by IFN-γ ELISpot of U90-specifc T cells in donor HD27 enriched using short term *in-vitro* reaction of PBMC with U90 pepmix.

PBMC from donor HD27 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Polyclonal T cells were either screened directly (A) or after enrichment of CD8+ T cells (B). Results are presented as spot-forming cells (SCF) per 10^5 T cells or CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. Cut off responses are indicated by dashed blue lines. (C) The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 2, 3 and 21 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptides 53 and 54 are highlighted in yellow. Positive pools 14, 15 and 22 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptides 82 and 83 are highlighted in yellow. (D) Flow cytometry analysis of polyclonal T cell populations (left hand panel) and CD8-enriched populations (right hand panel) used in the ELISpot analysis.

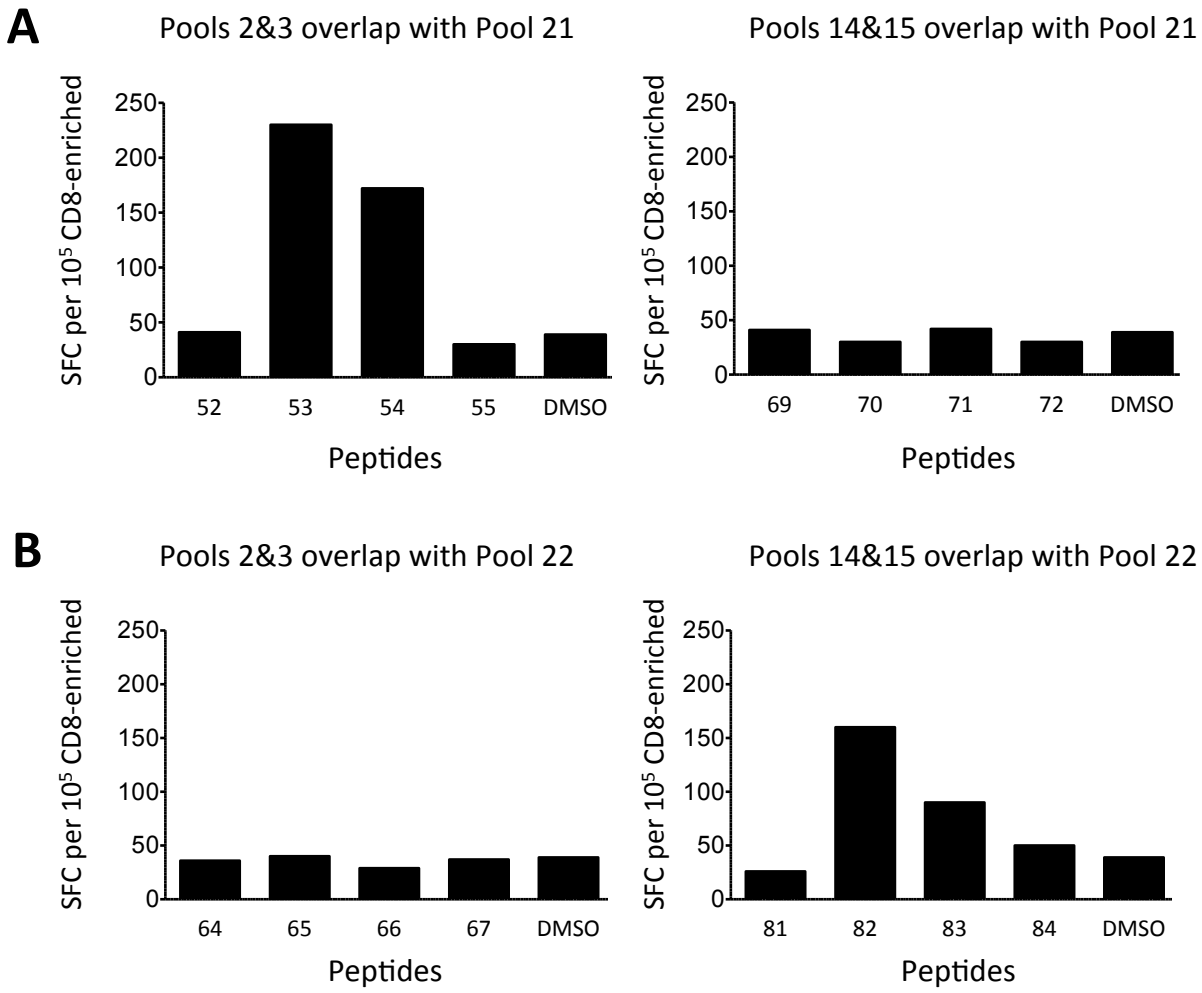


Figure 5.4 Confirmation of U90-specific T cell response to peptides 53 and 82 in donor HD27

(A) U90-specific polyclonal T cells from donor HD27, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 52, 53, 54 and 55 (left side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. 15-mer peptides 69, 70, 71 and 72 (right side) screened for Pool 2, 3 and Pool 22 were negative. (B) U90-specific polyclonal T cells from donor HD27, enriched by 10-day in-vitro reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 81, 82, 83 and 84 (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. 15-mer peptides 64, 65, 66 and 67 (left side) screened for Pool 14, 15 and Pool 21 were negative.

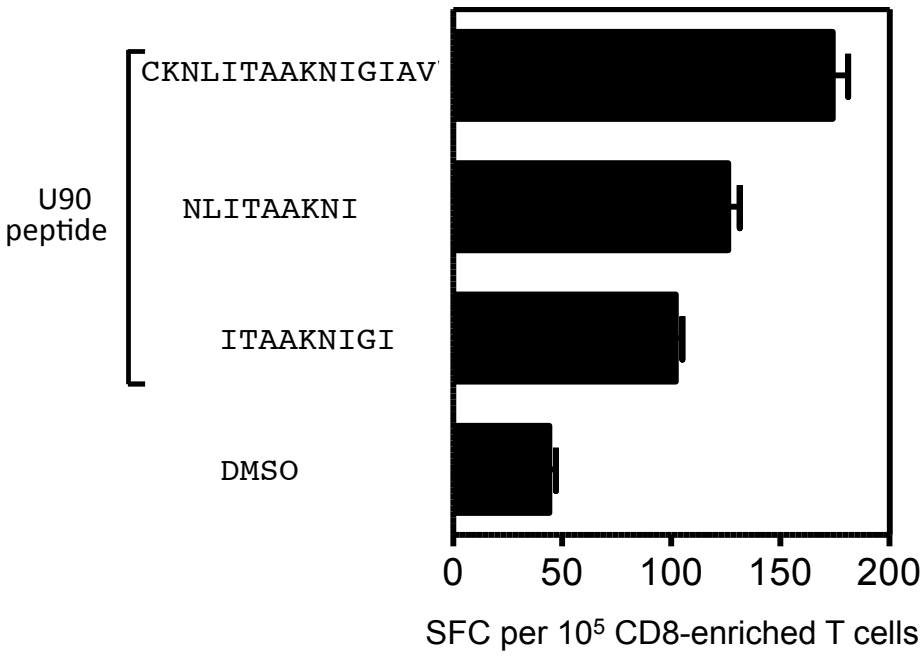


Figure 5.5 Mapping of minimal peptide epitope to NLITAAKNI and ITAAKNIGI in donor HD27

(A) U90 15-mer peptide 83 -specific polyclonal T cells from donor HD27, enriched by 10-day i*n-vitro* reactivation using 15-mer peptide 83, a CD4+ T cell depletion step was incorporated, to enrich for CD8+ T cells prior to screening by ELIspot 9-mers representing the 15-mer peptide 83 (CKNLITAAKNIGIAV). Using putative HLA-A2 binding peptides derived from the HHV6 U90 antigen, predicted using the SYFPEITHI epitope prediction programme, the minimal peptide epitope was mapped to the 9-mer peptides NLITAAKNI and ITAAKNIGI. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

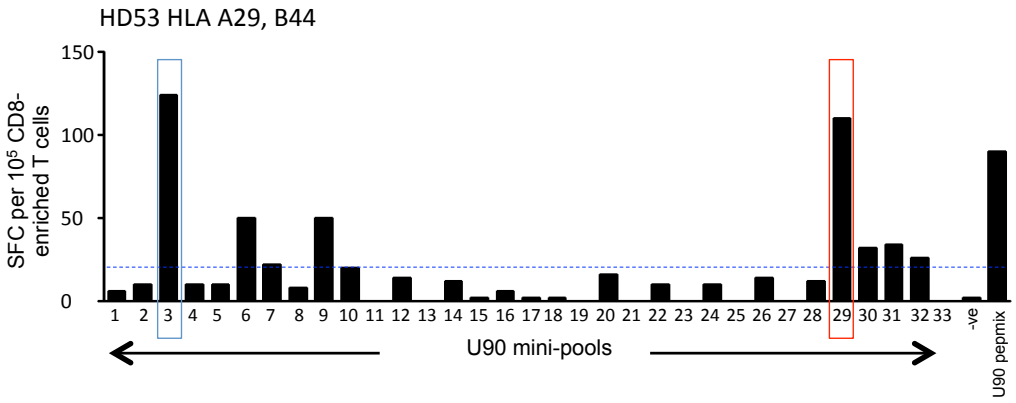
5.1.2 Mapping CD8+ T cell epitopes in donor HD53

Donor HD53 (HLA-A29, B44) also had strong response to U90 in pepmix expanded T cells (section 4.1.2). PBMC were stimulated with U90 pepmix, depleted of CD4 T cells and the CD8-enriched population screened with the 15-mer mini-pools (Figure 5.6). There were very strong responses direct against U90 mini-pools 3 and 29, with low levels seen against a small number of other pools (6, 9, 30, 31 and 32). Initially, responses to pools 3 and 29 were selected for further analysis. The intersect between these pools is highlighted in the 2-dimensional grid was the 15-mer peptide 190 (Figure 5.6). The enriched T cells were then screened by ELISpot against this 15-mer, and the 15-mers either side (peptides 189, 190 and 191). As shown in Figure 5.7A, T cells responded exclusively to peptide 190 (aa757–771, KPSKSKKIKLDRLPE), agreeing with the mini-pool screen data and implying that the minimal peptide was contained within this 15-mer sequence. Individual overlapping 9-mer peptides were then synthesised spanning the 15-mer sequence, and screened by ELIspot against the CD4-depleted polyclonal T cell line from donor HD53. Seven 9-mer peptides were tested, and a response was detected against a single peptide aa758-766, PSKSKKIKL (Figure 5.7B). This was deemed to be the minimal peptide epitope from U90, termed PSK, recognised by T cells from donor HD53 (this response is analysed further in sections 5.3 and 5.4).

CD8+ enriched T cell populations from donor HD53 were also screened against the intersecting (and flanking) peptides for pools 9 and 30, and 6 and 31. As seen in Figure 5.8 the 15-mer peptides 213 (aa849–863, IPIRSMPGTHDIRNK) and 227 (aa905-919, CFINHFVPIKTDDEE) were also identified as targets for T-cells from donor

HD57. Due to limited PBMC these were not followed up further, but the may represent novel U90 CD8+ T cell epitopes.

As this epitope was shown to be presented by HLA-A29 (see section 5.3, Figure 5.27) and a second donor was available carrying this allele (Donor HD33), a further experiment was undertaken. PBMC from donor HD33 were stimulated with the PSK peptide for 10 days to determine whether this donor also mounted a response to this peptide. ELISpot analysis of the expanded T cell culture is shown in Figure 5.9. Donor HD33 was found to respond to PSK. This supports the conclusion that this is a novel U90 CD8+ T cell epitope.



U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
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32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

Figure 5.6 U90-specifc T cells in donor HD53 enriched by short term *in-vitro* reaction of PBMC with U90 pepmix

PBMC from donor HD53 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 3 and 29 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptide 190 is highlighted in yellow.

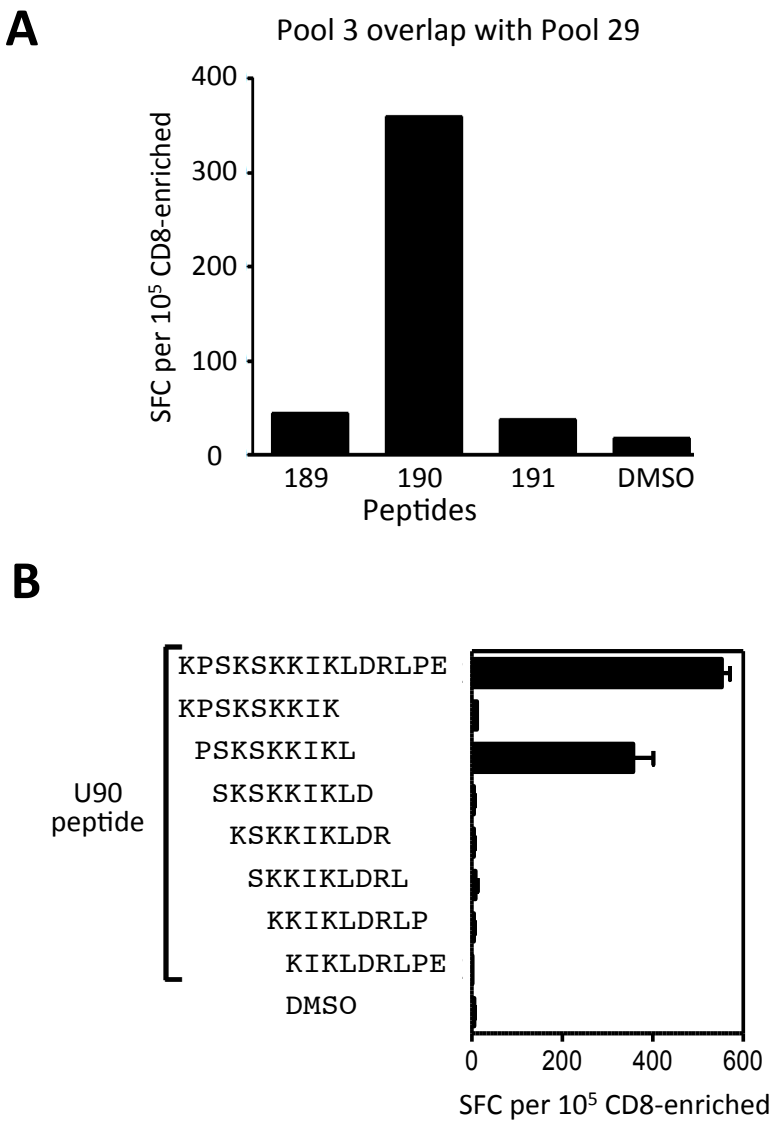


Figure 5.7 Confirmation of U90-specific T cell response to peptide 190 in donor HD53 and mapping of minimal peptide epitope to PSKSKKIKL

(A) U90-specific polyclonal T cells from donor HD53, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15mer peptides 189, 190 and 191. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. (B) Using overlapping 9mers representing the 15mer peptide 190 (KPSKSKKIKLDRLPE), the minimal peptide epitope was mapped by IFN-γ ELISpot to the 9mer peptide PSKSKKIKL.

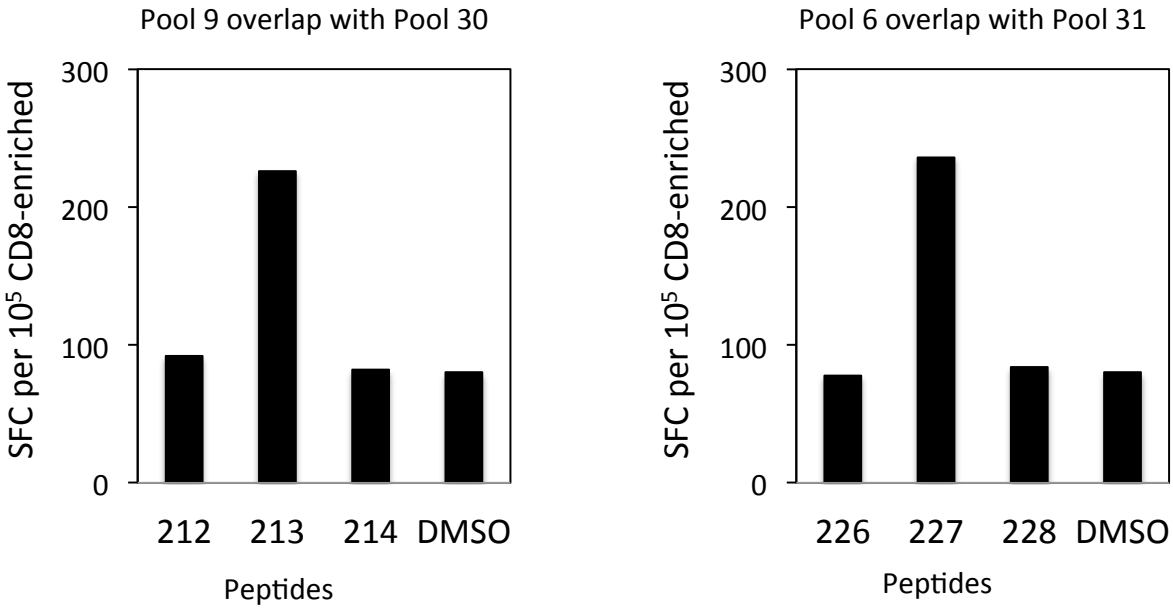


Figure 5.8 Confirmation of U90-specific T cell response to peptide 213 and 227 in donor HD53

U90-specific polyclonal T cells from donor HD53, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 212, 213 and 214 for Pool 9 and Pool 30 (left side). 15-mer peptides 226, 227 and 228 for Pool 6 and Pool 31 (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

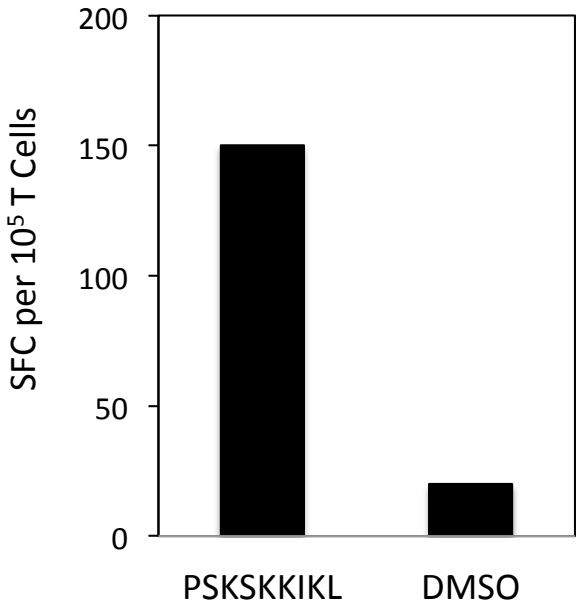


Figure 5.9 Confirmation of minimal peptide epitope to PSKSKKIKL in donor HD33

PBMC from donors HD33 were stimulated with PSKSKKIKL, and cultured for 10 days in the presence of IL2. HHV6B-specific polyclonal T cells were then screened by IFN-γ ELISpot. Results are presented as spot-forming cells (SFC) per 10^5 T cells. DMSO was used as negative control.

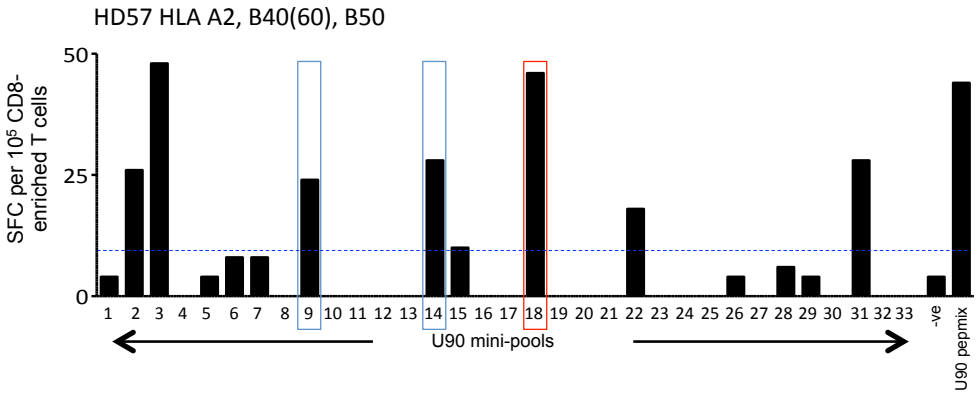
5.1.3 Mapping CD8+ T cell epitopes in donor HD57

PBMC from donor HD57 (HLA-A2, B40(60), B50) were reactivated with the U90 pepmix and a CD4-depleted polyclonal T cell line subsequently screened with the 15-mer mini-pools. A number of pools were found to give a response at least 2 times great than the BK-VP1 background, these were 2, 3, 9, 14, 18, 22 and 31 (Figure 5.10). In the first instance, responses to pools 9, 14 and 18 were selected for further analysis. These were selected based on unpublished data provided by Dr Ann Leen (Houston, USA) at the time that these experiments were been undertaken, identifying B40 restricted peptides corresponding to this region of U90 (this data was subsequently published, (Gerdemann *et al.,* 2013) – see discussion). The overlap regions for these pools, the 15-mer peptides 9 (overlap of 9 and 18), and 14, (overlap of 14 and 18) are highlighted in the 2 dimension matrix in Figure 5.10. To confirm these peptides contained a CD8+ T cell epitope, the T cell line was then screened by ELISpot against these 15-mer peptides, and the relevant flanking 15-mers. Data shown in Figure 5.11 shows that the 15-mer peptide 9 (Figure 5.11A, left hand panel) and 15-mer peptides 14 and 15 (Figure 5.11B, left hand panel) are recognised by these T cells from donor HD57. Peptide 9 (aa33–47; YHPDPVVEESIKEIL) includes the sequence VEESIKEIL (aa39-47, VEE) identified by Leen and colleagues (Gerdemann *et al.,* 2013) as a HLA-B40 restricted peptide in HHV6B U90. The VEE peptide was thus tested on Donor HD57 T cells, and found to induce a response in ELISpot (Figure 5.11A, right hand panel). The lack of response to peptide 10 that also includes the sequence VEESIKEIL is due to the processing deficiency that comes with using non-professional APCs as presenters (Gerdemann *et al.,* 2013).

The overlap of 15-mer peptides 14 (aa53-67; CDVSFESLLFPELEA) and 15 (aa57-71 (FESLLFPELEAFDLF) contained the peptide FESLLFPEL (aa57-65, FES). This was also

identified by Leen and colleagues as a HLA-B40 restricted peptide epitope in HHV6B U90 (Gerdemann *et al*., 2013). As before, expanded T cells from donor HD57 were tested against FES by Elispot and found to respond to this peptide (Figure 5.11B, right hand panel). Two additional HLA-B40 positive donors [HD05 HLA-A2, A24, B40(60), B44 and HD30 HLA-A2, A32, B7, B40(60)] were also screened for responses to VEE and FES. PBMC were reactivated with each 9-mer, and after 10 day culture tested by ELISpot. Both donor HD05 and HD30 were found to respond to these B40-restricted peptide in U90 (Figure 5.12).

The other positive pools from the mini-pool screen of HD57 U90 pepmix stimulated cells, polls 2, 3, 20, and 31 (Figure 5.10) were not followed up but may represent an additional U90 T cell epitope recognised by this donor.



U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
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32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
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Figure 5.10 Screening of U90-specifc T cells in donor HD57 enriched by short term *in-vitro* reaction of PBMC with U90 pepmix

PBMC from donor HD57 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 9, 14, and 18 are highlighted in blue and red. The 15-mer peptide present in overlapping pools 9 and 18, peptide 9, and pools 14 and 18, peptide 14 are highlighted in yellow.

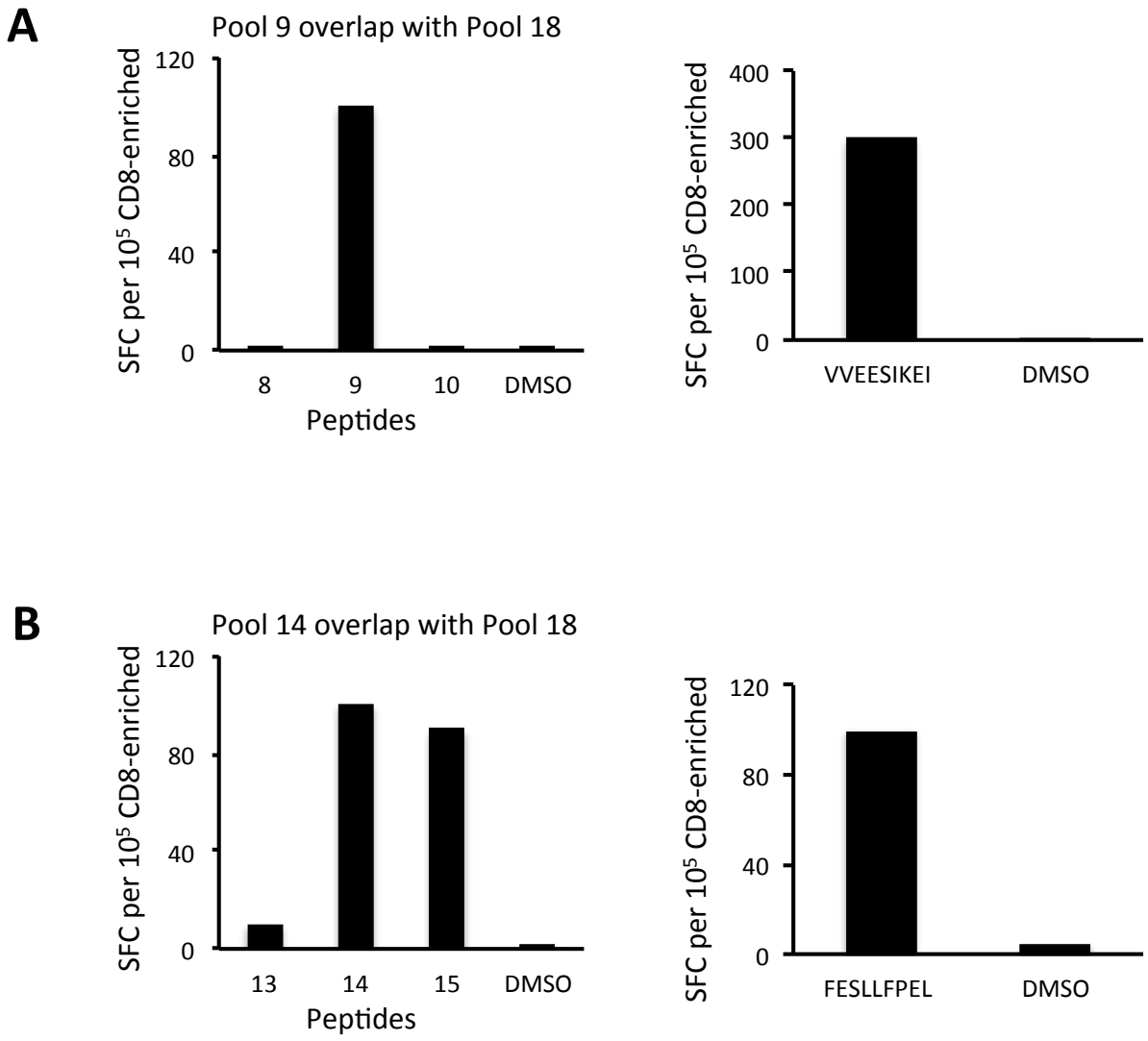


Figure 5.11 Confirmation of U90-specific T cell response to peptides 9 and 14 in donor HD57 and mapping of minimal peptide epitope to VEESIKEIL and FESLLFPEL

(A) U90-specific polyclonal T cells from donor HD53, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 8, 9 and 10 (left side). Confirmation of the U90-specific CD8+ T cell response to the individual 9-mer peptide VEESIKEIL (right side). (B) U90-specific polyclonal T cells from donor HD53, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 13, 14 and 15 (left side). Confirmation of the U90-specific CD8 T cell response to the individual 9-mer peptide FESLLFPEL (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

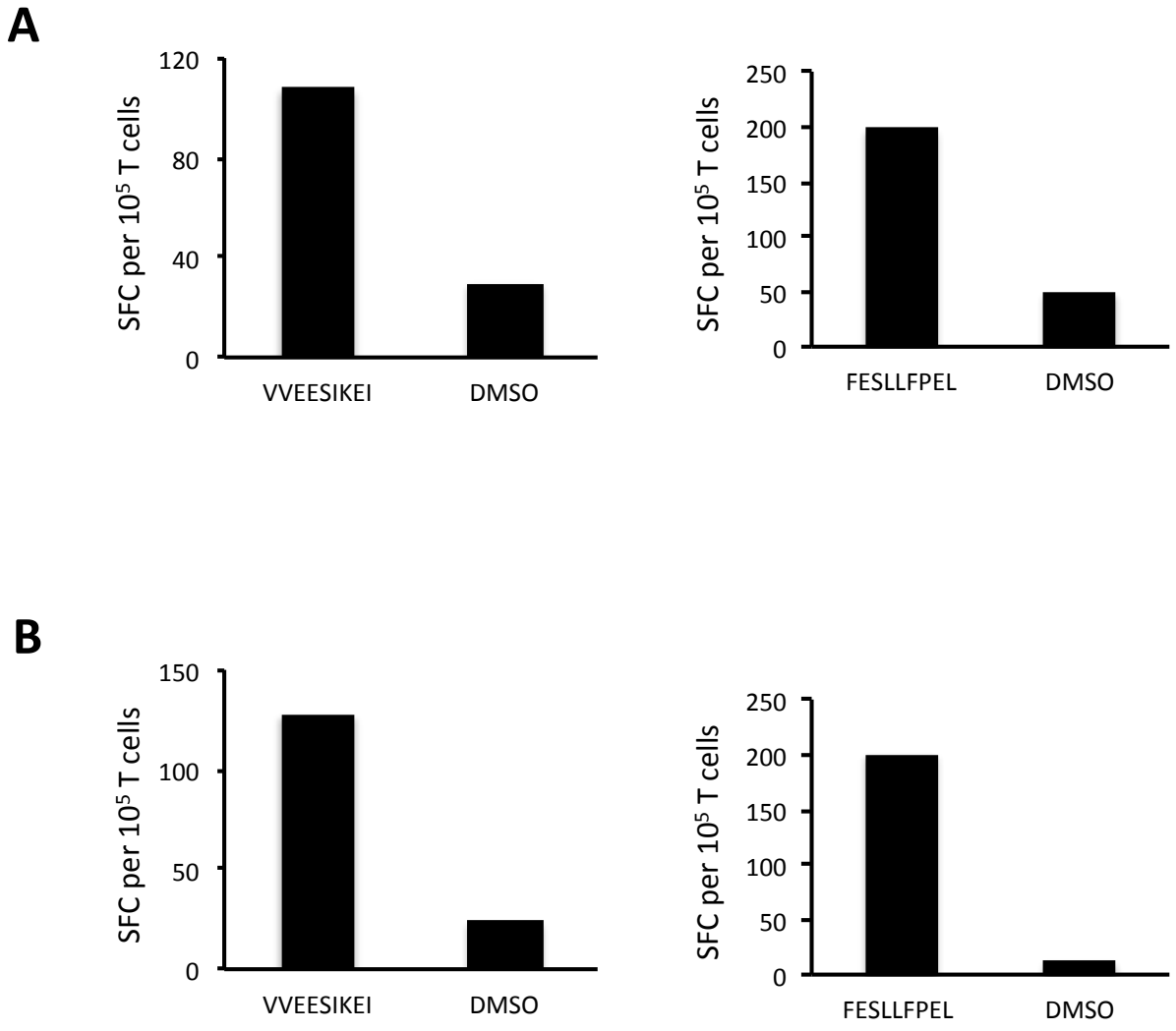
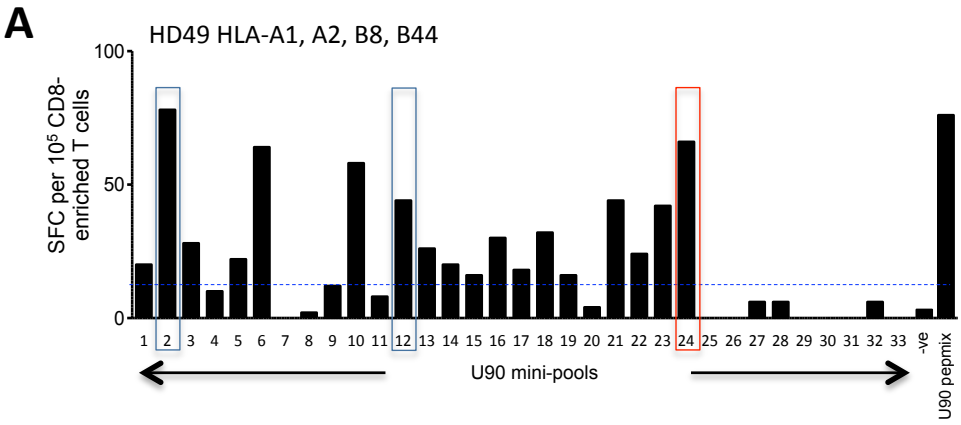


Figure 5.12 Confirmation of minimal peptide epitope to VEESIKEIL and FESLLFPEL in donors HD05 and HD30

(A) PBMC from donors HD05 were stimulated with VEESIKEIL (left side) and FESLLFPEL (right side), then cultured for 10 days in the presence of IL2. HHV6B-specific polyclonal T cells were then screened by IFN-γ ELISpot. Results are presented as spot-forming cells (SCF) per 10^5 T cells. DMSO was used as negative control. (B) PBMC from donors HD30 were stimulated with VEESIKEIL (left side) and FESLLFPEL (right side), then cultured for 10 days in the presence of IL2. HHV6B-specific polyclonal T cells were then screened by IFN-γ ELISpot. Results are presented as spot-forming cells (SCF) per 10^5 T cells. DMSO was used as negative control.

5.1.4 Mapping CD8+ T cell epitopes in donor HD49

The fourth donor to be analysed was HD49 (HLA-A1, A2, B8, B44). PBMC were reactivated with the U90 pepmix and a CD4-depleted polyclonal T cell line subsequently screened with the 15-mer mini-pools. The results are shown in Figure 5.13. There were a large number of U90 mini-pools above the cut-off line. Those considered positive were pools 2, 3, 6, 10, 12, 16, 18, 21, 22, 23 and 24. Initially, responses to pools 2, 12 and 24 were selected for further analysis. The overlap regions for these pools are highlighted in the 2 dimension matrix, the 15-mer peptides 104 and 114 (Figure 5.13). Theses individual 15-mers and flanking peptides were then tested in ELISpot against CD4-depleted polyclonal T cell line. T cells responded to peptides 104 (aa413–427 SICDLNIDPSESILL) and 114 (aa453-467 NVDYLKYMEVQSPTD) (Figure 5.14A), agreeing with the mini pool screen data. Individual overlapping 9-mer peptides were then synthesised spanning the 15-mer peptide 104 (SICDLNIDPSESILL) sequence, and screened by ELIspot against the CD4-depleted polyclonal T cell line from donor HD49. A response was detected against a single 9-mer peptide, aa417–425, LNIDPSESI (Figure 5.14B). This was deemed to be the a CD8+ peptide from U90, termed LNI, recognised by T cells from donor HD49. The response to the individual 15-mer peptide 114 (NVDYLKYMEVQSPTD) was very weak (Figure 5.14A), and due to limiting T cell numbers this was not followed up, although it may represent a target epitope with U90. No responses were confirmed to the other 15-mer mini-pool hits.



U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
20	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
21	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
22	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85
23	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
24	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
25	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136
26	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
27	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
28	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187
29	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204
30	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221
31	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

Figure 5.13 U90-specifc T cells in donor HD49 enriched by short term *in-vitro* reaction of PBMC with U90 pepmix

PBMC from donor HD49 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 2, 12 and 24 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptides present in these pools, peptides 104 and 114 are highlighted in yellow.

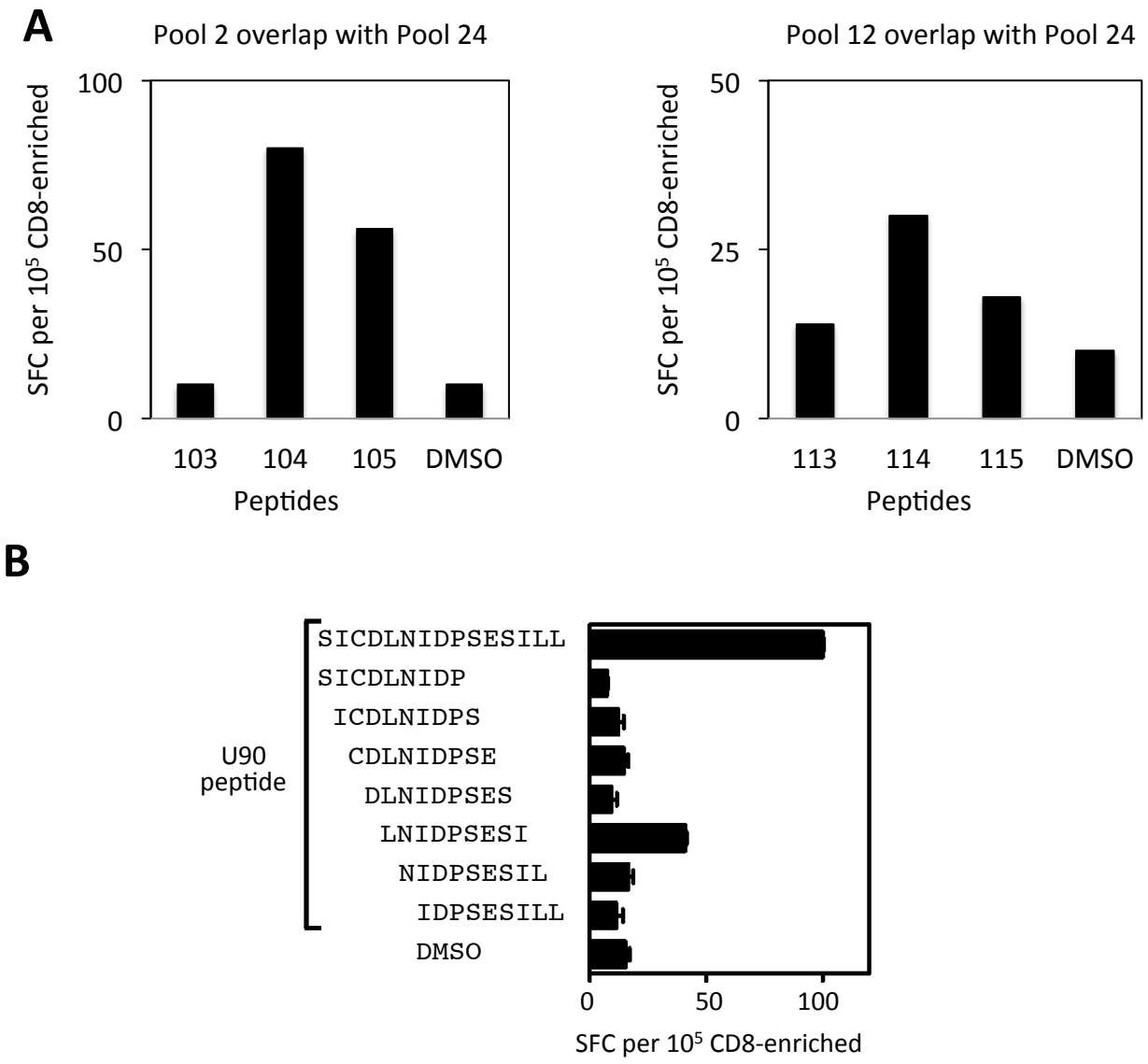
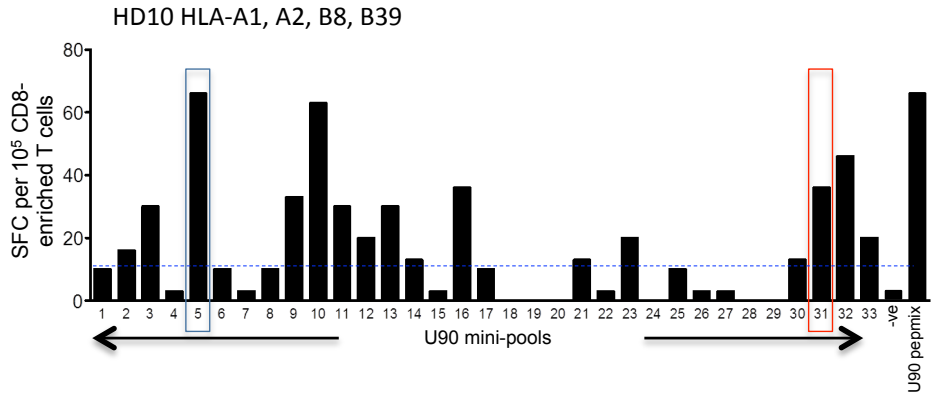


Figure 5.14 Confirmation of U90-specific T cell response to peptide 104 in donor HD49 and mapping of minimal peptide epitope to LNIDPSESI

(A) U90-specific polyclonal T cells from donor HD49, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 103, 104 and 105 for Pool 2 and Pool 24 (left side). 15-mer peptides 113, 114 and 115 for Pool 12 and Pool 24 (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells (B) Using overlapping 9-mers representing the 15-mer peptide 104 (SICDLNIDPSESILL), the minimal peptide epitope was mapped by IFN-γ ELISpot to the 9-mer peptide LNIDPSESI.

5.1.5 Mapping CD8+ T cell epitopes in donor HD10

The fifth donor to be analysed was HD10 (HLA-A1, A2, B8, B39). The results from the 15-mer mini pools screen on a CD4-depleted T cell line are shown in Figure 5.15. A large number of U90 mini-pools were above the cut-off, and considered positive. These were pools 2, 3, 5, 9, 10, 11, 12, 16, 18, 21, 23, 31, 32 and 33. Initially, responses to pools 5 and 31 were selected for further analysis, the intersect between these pools is the 15-mer peptide 226 (Figure 5.15). To confirm this peptide contained a CD8+ T cell epitope, the T cell line was then screened by ELISpot against this 15-mer, and the 15-mers either side (i.e. peptides 225, 226 and 227). As shown in Figure 5.16A, the response to the individual 15-mers was weak, although peptide 226 (aa901–915, EANHCFINHFVPIKT) gave the strongest response above the other 15-mers and DMSO control. Although weak, this response was analysed further. Individual overlapping peptides representing this sequence were synthesised and screened by ELIspot against the CD4-depleted polyclonal T cell line from donor HD10. Seven peptides were tested, in general the response was low, but two peptides (ANHCFINHFV and NHCFINHFV) were recognised at equivalent levels (Figure 5.15B). As loss of the alanine residue appeared to have little effect on recognition, whereas loss of the asparagine and also the valine has a significant effect. Thus, it was deemed to be the minimal peptide epitope from U90 recognised by donor HD10 was the 9-mer peptide aa903–911, NHCFINHFV, termed NHC (Figure 5.16B). From representative experiments with donor HD10, two other 15-mer peptides were identified as targets for T-cell responses (Figure 5.17). The potential response to the 15-mer peptides 231 aa921–935 (EKENVSYTYSKIQDS) and 254 aa1013-1027 (GNNSPILNIINDTTC) was not followed up but may represent an additional U90 T cell epitope recognised by this donor.



U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
20	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
21	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
22	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85
23	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
24	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
25	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136
26	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
27	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
28	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187
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31	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

Figure 5.15 U90-specifc T cells in donor HD10 enriched by short term *in-vitro* reaction of PBMC with U90 pepmix

PBMC from donor HD10 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 5 and 31 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptide 226 is highlighted in yellow.

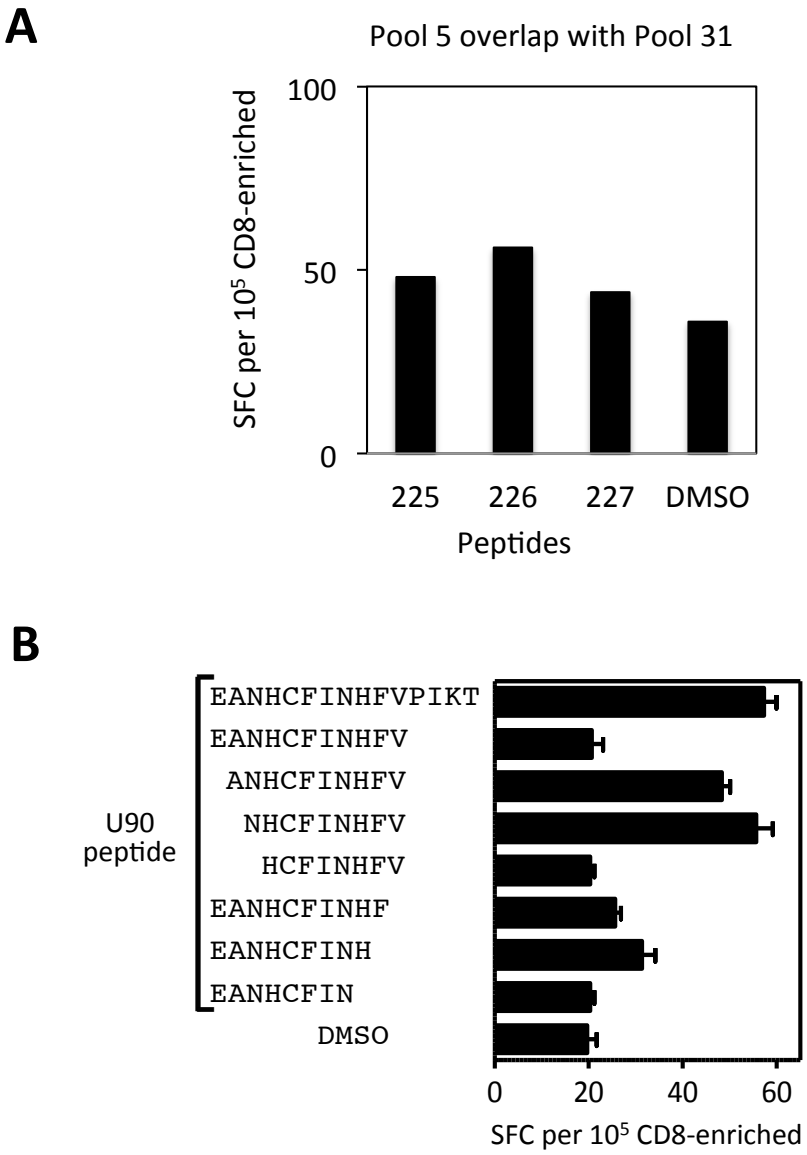


Figure 5.16 Confirmation of U90-specific T cell response to peptide 226 in donor HD10 and mapping of minimal peptide epitope to NHCFINHFV

(A) U90-specific polyclonal T cells from donor HD10, enriched by 10-day *in-vitro* reactivation using a U90 pepmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 103, 104 and 105. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. (B) Using overlapping 9-mers representing the 15-mer peptide 226 (EANHCFINHFVPIKT), the minimal peptide epitope was mapped by IFN-γ ELISpot to the 9-mer peptide NHCFINHFV.

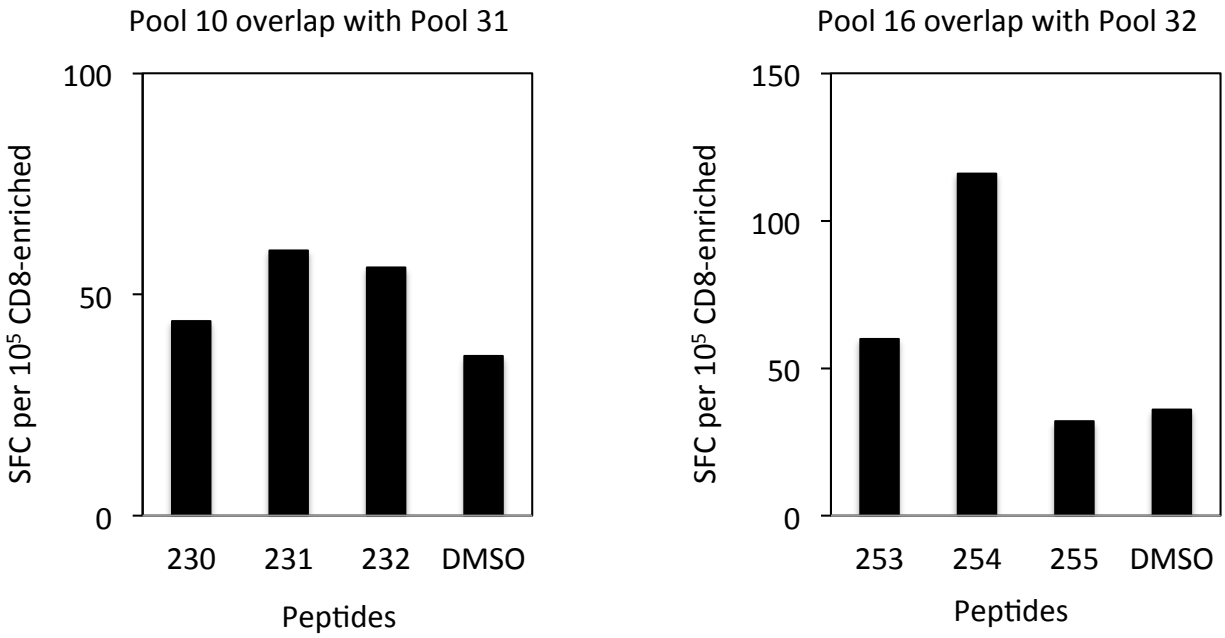
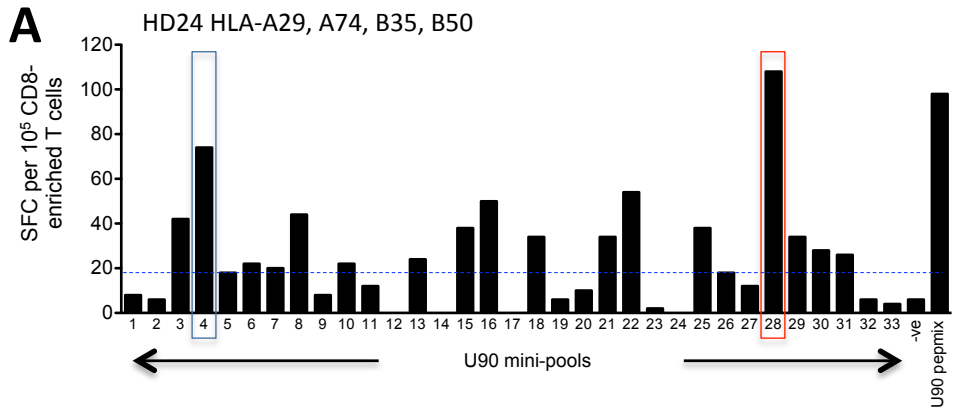


Figure 5.17 Confirmation of U90-specific T cell response to peptides 231 and 254 in donor HD10

U90-specific polyclonal T cells from donor HD10, enriched by 10-day *in-vitro* reactivation using a U90 peptmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 230, 231 and 232 for Pool 10 and Pool 31 (left side). 15-mer peptides 253, 254 and 255 for Pool 16 and Pool 32 (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

5.1.6 Mapping CD8+ T cell epitopes in donor HD24

The last donor to be analysed for responses to U90 was HD24 whose HLA type was HLA-A29, A74, B35, B50. The results from the 15-mer mini pools screen on a CD4-depleted T cell line are shown in Figure 5.18. U90 mini-pools which were considered positive for HD24 were pools 3, 4, 8, 13, 15, 16 and 18, 21, 22, 25, 28, 29, 30, 31. Pools 4 and 28 had the strongest response and were selected for further analysis. The intersect between these pools was the 15-mer peptide 174 (Figure 5.18). To confirm this peptide contained a CD8+ T cell epitope, the T cell line was then screened by ELISpot against peptides 173, 174 and 175. As shown in Figure 5.18B, T cells responded to peptide 173 (aa689–703 YIADCDSTVRTNNIH and 174 (aa693–707 CDSTVRTNNIHMNNT), with the strongest response to peptide 174, suggesting that the minimal peptide was contained within this 15-mer sequence. However, this donor was simultaneously analysed for U11 response, and subsequently was not possible to follow this donor up further.



U90	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
20	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
21	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
22	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85
23	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
24	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
25	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136
26	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
27	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
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30	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221
31	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
32	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
33	256	257	258	259	260	261	262	263	264	265	266	267					

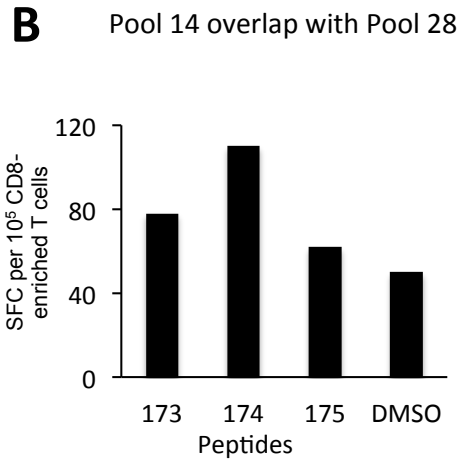


Figure 5.18 U90-specifc T cells in donor HD24 enriched by short term *in-vitro* reaction of PBMC with U90 pepmix and confirmation peptide 174 response

PBMC from donor HD24 were stimulated with U90 pepmix, and cultured for 10 days in the presence of IL2. U90-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U90 protein, in 33 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U90 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 33 pools of U90 15-mer peptides used in the screen. Positive pools 4 and 28 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptide 174 is highlighted in yellow. (B) U90-specific polyclonal T cells from donor HD24, enriched by 10-day *in-vitro* reactivation using a U90 peptmix, were then screened by IFN-γ ELISpot against U90 15-mer peptides 173, 174 and 175 for Pool 4 and Pool 28.

5.2 Mapping of HLA Class I restricted T cell epitopes in HHV6B antigen U11

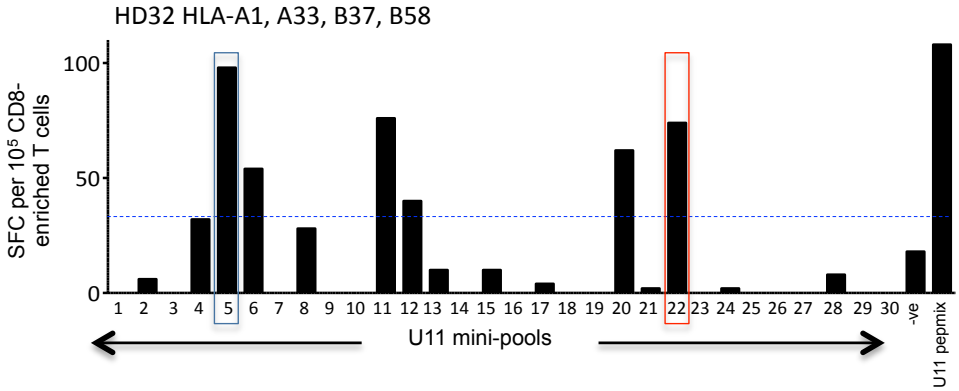
U11-specific T cells were identified in expanded T cell populations in 19 (out of 25) donors (see section 4.1.1). In this section, 3 were selected for further analysis in this chapter. These were donors HD32, HD24, and HD57. Both HD24 and HD57 responded to all four HHV6B antigens tested. Whereas, HD32 was shown to respond to U11 and U54. PBMC were isolated, stimulated with U11 pepmixes in the presence of IL2 for 10 days, and subsequently analysed with the 15-mer mini-pools. In some cases, PBMC were subsequently stimulated with individual 15-mers to allow for follow up mapping experiments.

5.2.1 Mapping CD8+ T cell epitopes in donor HD32

HD32 (HLA-A1, A33, B37, B58) was the first donor analysed for U11 responses. The results from the 15-mer mini pools screen on a CD4-depleted T cell line are shown in Figure 5.19. U11 mini-pools which were considered positive were pools 5, 6, 11, 12, 20 and 22. Responses to pools 5 and 22 were selected for further analysis, the intersect between these pools was the 15-mer peptide 85 (Figure 5.19). To confirm this peptide contained a CD8+ T cell epitope, the T cell line was then screened by ELISpot against this 15-mer, and the 15-mers either side (i.e. peptides 84, 85 and 86). As shown in Figure 5.20, T cells responded to peptide 85 (aa337–351; PLKTQRRHKFPESDS). Individual overlapping 9-mer peptides were then synthesised spanning the 15-mer sequence, and screened by ELIspot against the CD4-depleted polyclonal T cell line from donor HD32. Seven 9-mer peptides were tested, and the highest response was detected against a 9-mer peptide aa338-346, LKTQRRHKF

(Figure 5.20B). This was deemed to be the minimal peptide epitope from U11, termed LKT after the first three amino acids, recognised by T cells from donor HD32.

From same representative experiment with donor HD32, the 15-mer peptides 59 (aa233–247 RKNENFNAVYSQRVL) and 88 (aa349-363 SDSVDNAGGKILIKK) were also identify as target for T-cell response (Figure 5.21A). As shown in Figure 5.21B, T cells responded to peptide 59 aa233–247 (RKNENFNAVYSQRVL), agreeing with the mini pool screen data and implying that the minimal peptide was contained within this 15-mer sequence. Individual overlapping 9-mer peptides were then synthesised spanning the 15-mer sequence, and screened by ELIspot against the CD4-depleted polyclonal T cell line from donor HD32. Seven 9-mer peptides were tested, and the highest response was detected against a 9-mer peptide aa238-246 FNAVYSQRV (Figure 5.21B). This was deemed to be the minimal peptide epitope from U11, termed FNA after the first three amino acids, recognised by T cells from donor HD32. However, the potential T cells response to the 15-mer peptide 88 aa349-363 (SDSVDNAGGKILIKK) was not followed up.



U11	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
17	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
18	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
19	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
20	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
21	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
22	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
23	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
24	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
25	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
26	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
27	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
28	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
29	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
30	209	210	211	212												

Figure 5.19 U11-specifc T cells in donor HD32 enriched by short term *in-vitro* reaction of PBMC with U11 pepmix

PBMC from donor HD32 were stimulated with U11 pepmix, and cultured for 10 days in the presence of IL2. U11-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U11 protein, in 30 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U11 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 30 pools of U11 15-mer peptides used in the screen. Positive pools 5 and 22 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptide 85 is highlighted in yellow.

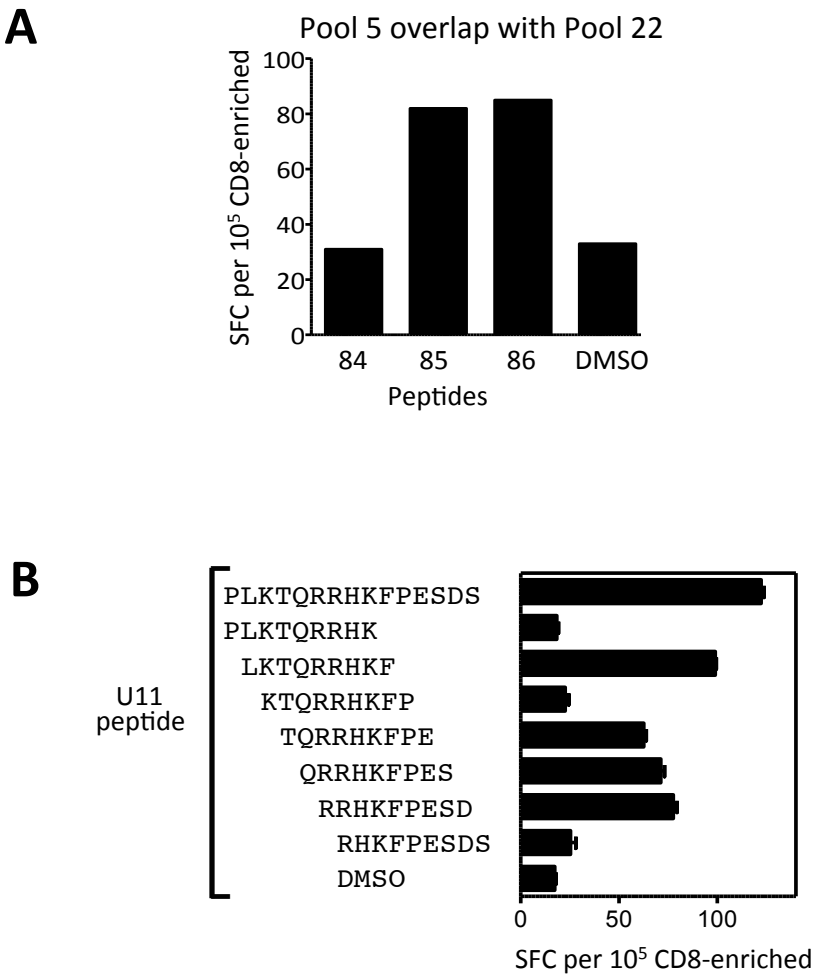


Figure 5.20 Confirmation of U11-specific T cell response to peptide 85 in donor HD32 and mapping of minimal peptide epitope to LKTQRRHKF

(A) U11-specific polyclonal T cells from donor HD32, enriched by 10-day *in-vitro* reactivation using a U11 pepmix, were then screened by IFN-γ ELISpot against U11 15-mer peptides 84, 85 and 86. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. (B) Using overlapping 9-mers representing the 15-mer peptide 85 (PLKTQRRHKFPESDS), the minimal peptide epitope was mapped by IFN-γ ELISpot to the 9-mer peptide LKTQRRHKF. Note, there may be responses to two minimal epitopes identified here.

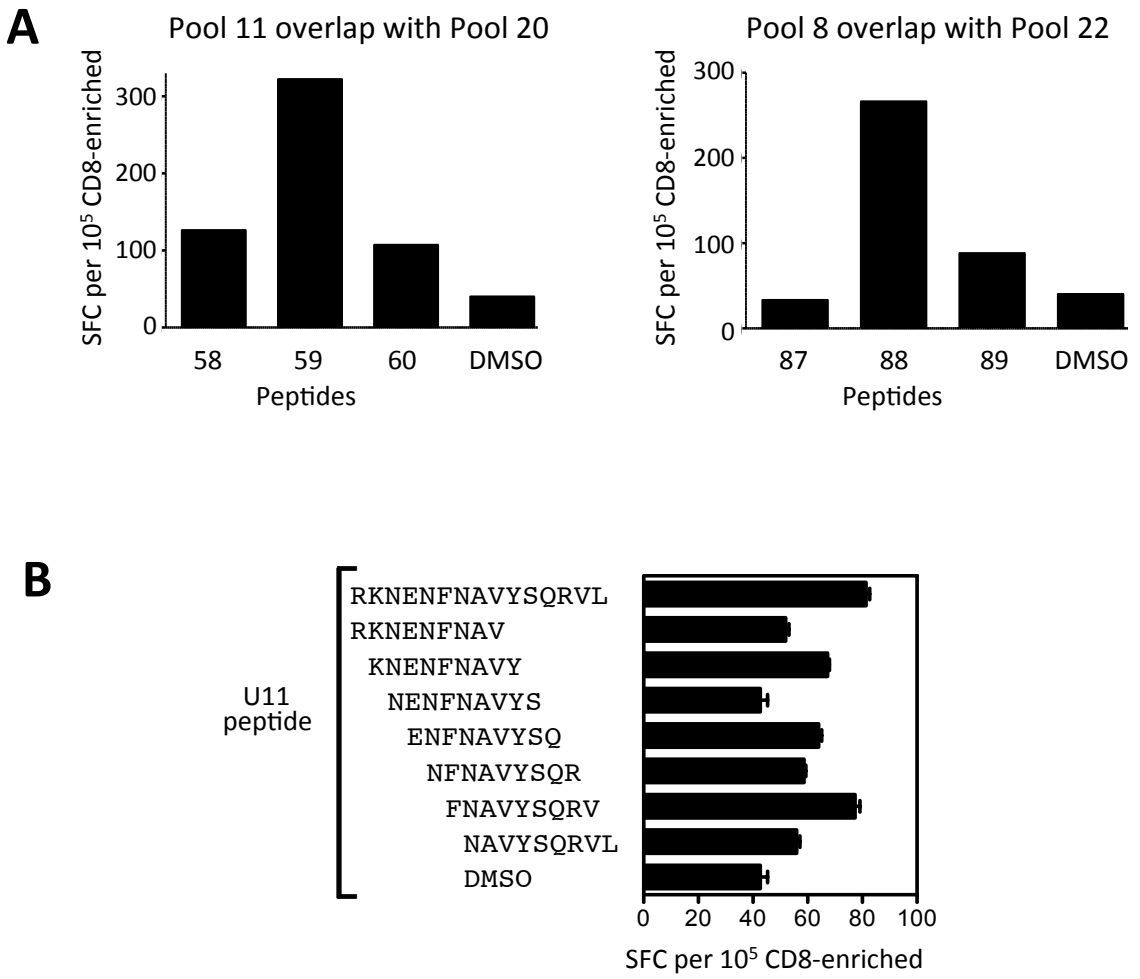


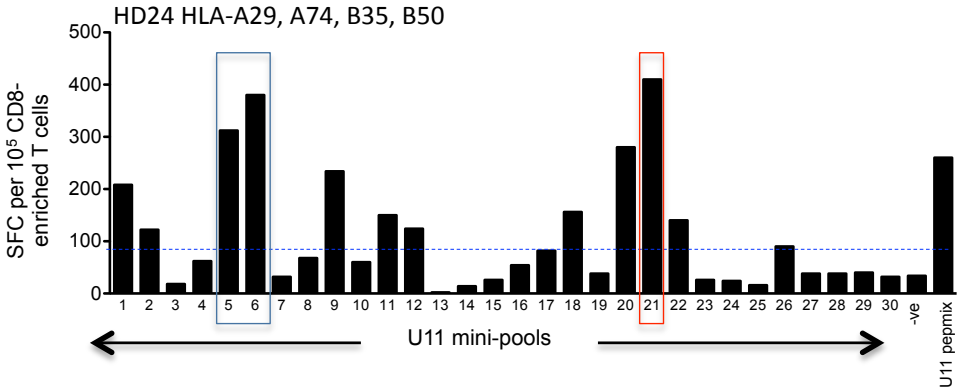
Figure 5.21 Confirmation of U11-specific T cell response to peptides 59 and 88 in donor HD32 and mapping of minimal peptide epitope to FNAVYSQRV

(A) U11-specific polyclonal T cells from donor HD32, enriched by 10-day *in-vitro* reactivation using a U11 pepmix, were then screened by IFN-γ ELISpot against U11 15-mer peptides 58, 59 and 60 for Pool 11 and Pool 20 (left side). 15-mer peptides 87, 88 and 89 for Pool 8 and Pool 22 are (right side). DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. (B) Using overlapping 9-mers representing the 15-mer peptide 59 (RKNENFNAVYSQRVL), the minimal peptide epitope was mapped by IFN-□ ELISpot to the 9-mer peptide FNAVYSQRV.

5.2.2 Mapping CD8+ T cell epitopes in donor HD24

The second donor to be analysed was HD24 (HLA-A29, A74, B35, B50). The results from the 15-mer mini pools screen on a CD4-depleted T cell line are shown in Figure 5.22. U11 mini-pools which were considered positive for HD24 were pools 1, 2, 5, 6, 9, 11, 12, 18, 20, 21, 22 and 26. There were very strong responses directed against pools 5, 6 and 21 and these were selected for initial further analysis. The intersect between these pools were the 15-mer peptides 69 (pools 5 and 21) and 70 (pools 6 and 21) (Figure 5.22). As the peptides were adjacent to each other, a T cell line was then screened by ELISpot against these 15-mers, and the two flanking 15-mers. As shown in Figure 5.23A, T cells responded to both peptide 69 (aa273–287; SLESYSASKAFSVPE), and peptide 70 (aa277–291; YSASKAFSVPENGPH), but not the flanking peptides.

From same representative experiment with donor HD24, the 15-mer peptides 17 (aa65–79 ILWLMYHYVLSKRKP), 49 (aa193–207 INKLVYLGKLFVTLN) and 89 (aa353-367 DNAGGKILIKKETLG) were also identified as targets for T-cell responses in the same way (Figure 5.23 B, C and D respectively).



U11	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
17	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
18	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
19	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
20	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
21	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
22	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
23	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
24	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
25	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
26	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
27	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
28	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
29	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
30	209	210	211	212												

Figure 5.22 U11-specifc T cells in donor HD24 enriched by short term *in-vitro* reaction of PBMC with U11 pepmix

PBMC from donor HD24 were stimulated with U11 pepmix, and cultured for 10 days in the presence of IL2. U11-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U11 protein, in 30 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U11 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 30 pools of U11 15-mer peptides used in the screen. Positive pools 5, 6 and 21 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptides present these pools, peptide 69 and 70 is highlighted in yellow.

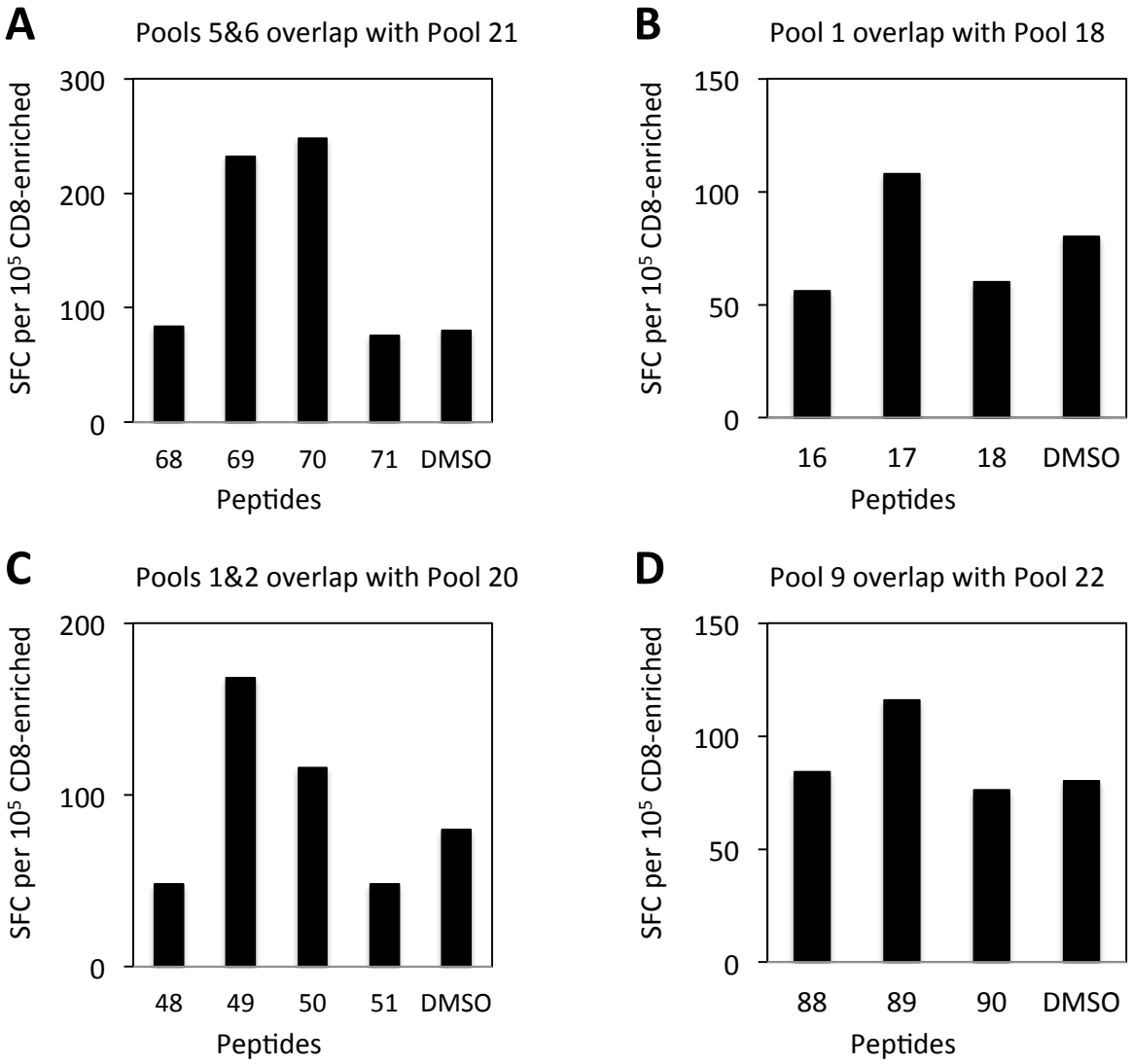
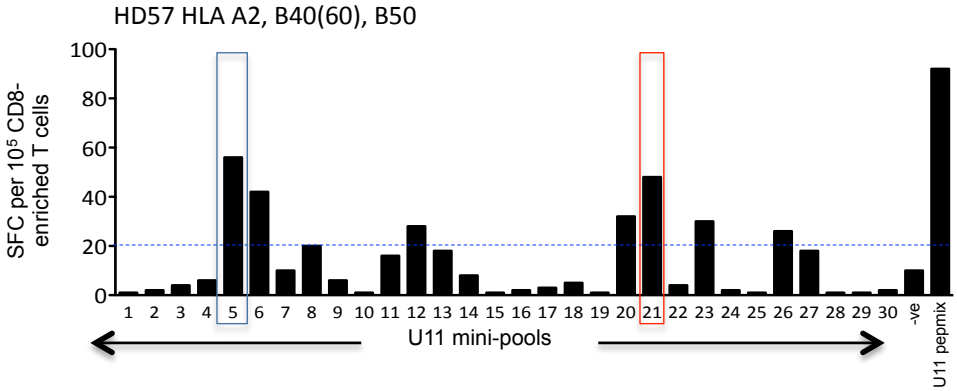


Figure 5.23 Confirmation of U11-specific T cell response to peptides 17, 49, 70 and 89 in donor HD24

(A) U11-specific polyclonal T cells from donor HD24, enriched by 10-day *in-vitro* reactivation using a U11 pepmix, were then screened by IFN-γ ELISpot against U11 15-mer peptides 68, 69, 70 and 71 for Pools 5, 6 and Pool 21, (B) 15-mer peptides 16, 17 and 18 for Pool 1 and Pool 18, (C) 15-mer peptides 48, 49, 50 and 51 for Pools 1, 2 and Pool 20 and (D) 15-mer peptides 88, 89 and 90 for Pool 9 and Pool 22. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

5.2.3 Mapping CD8+ T cell epitopes in donor HD57

The final donor analysed was HD57 (HLA-A2, B40(60), B50). The results from the 15-mer mini pools screen on a CD4-depleted T cell line are shown in Figure 5.24. U11 mini-pools which were considered positive were pools 5, 6, 12, 20, 21, 23 and 26. To confirm these peptides contained a CD8+ T cell epitope, the T cell line was then screened by ELISpot against these 15-mers, and the appropriate flanking peptides. T cells responded to peptide 69 (aa273–287; SLESYSASKAFSVPE), peptide 104 (aa413-427; GILDFGVKLPAEAQS) and peptide 60 (aa237–251; NFNAVYSQRVLQTPL), with minimal recognition of any flanking peptides. (Figure 5.25A, B and C respectively). The peptide 104 includes the sequence GILDFGVKL (aa413-421, GIL), which while these experiments were underway was identified as a HLA-A2 restricted peptide in HHV6B U11 (Martin *et al.,* 2012). The expaneded T cell population from donor HD57 was tested and shown to recognise the GIL peptide (Figure 5.25C right hand panel).



U11	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
17	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
18	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
19	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
20	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
21	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
22	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
23	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
24	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
25	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
26	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
27	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
28	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
29	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
30	209	210	211	212												

Figure 5.24 U11-specifc T cells in donor HD57 enriched by short term *in-vitro* reaction of PBMC with U11 pepmix

PBMC from donor HD57 were stimulated with U11 pepmix, and cultured for 10 days in the presence of IL2. U11-specific polyclonal T cells were then screened by IFN-γ ELISpot against overlapping 15-mer peptides, representing the entire U11 protein, in 30 pools such that each 15-mer was present in two pools. Polyclonal T cells were enriched for CD8+ T cells. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells. Pepmixes representing U11 and BK-VP1 were used as positive and negative control respectively. The lower grid represents the 30 pools of U11 15-mer peptides used in the screen. Positive pool 5 and 21 (based on cut off line) are highlighted (blue and red respectively), and the 15-mer peptide present in both these pools, peptide 69 is highlighted in yellow.

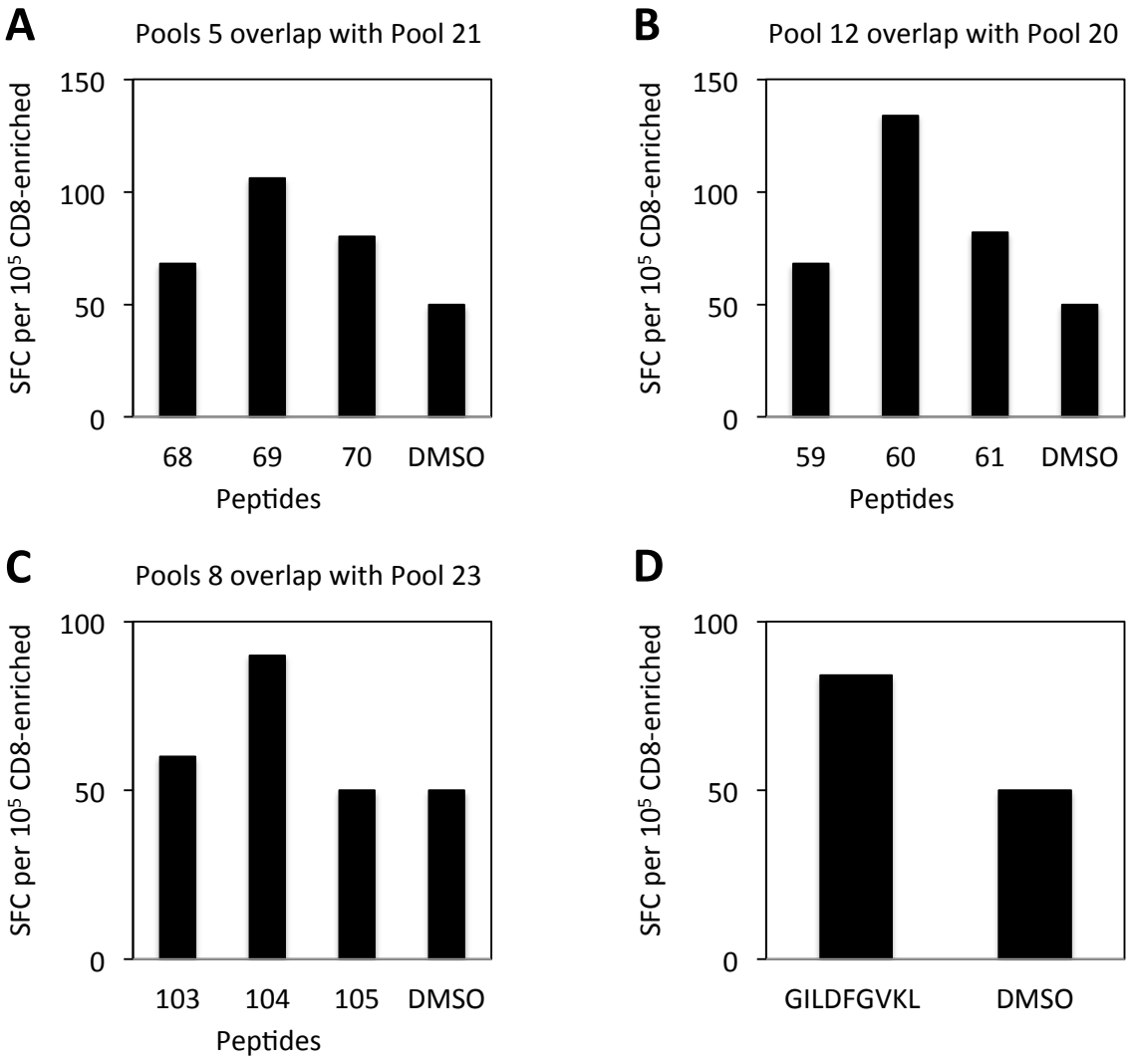


Figure 5.25 Confirmation of U11-specific T cell response to peptides 60, 69 and 104 and confirmation of minimal peptide epitope to GILDFGVKL in donor HD57

(A) U11-specific polyclonal T cells from donor HD24, enriched by 10-day *in-vitro* reactivation using a U11 pepmix, were then screened by IFN-γ ELISpot against U11 15-mer peptides 68, 69 and 70 for Pools 5 and Pool 21, (B) 15-mer peptides 59, 60 and 61 for Pool 12 and Pool 20, (C) 15-mer peptides 103, 104 and 105 for Pool 8 and Pool 23. (D) Confirmation of the U90-specific CD8 T cell response to the individual 9-mer peptide GILDFGVKL. DMSO pulsed cells were used as a negative control. Results are presented as spot-forming cells (SCF) per 10^5 CD8-enriched T cells.

5.3 Characterisation of HLA restriction elements for antigenic peptide identified in U90 and U11

To determine the HLA restricting alleles of newly identified HHV6B peptide epitopes, autologous, partially HLA-matched and HLA mis-matched PHA-blasts (or PBMC) were used as targets in T-cell assays. T cell populations were generated by 10-day culture of donor PBMC stimulated with individual 9-mer peptides mapped in section 5.2 (peptides U90 FESLLFPEL, NLITAAKNI, ITAAKNIGI, LNIDPSESI, PSKSKKIKL, NHCFINHFV and U11 LKTQRRHKF). Polyclonal cultures were then used as effectors in T cell assays. Appropriate target cells were loaded with individual peptides and incubated with an Effector-to-Target (E:T) ratio of 10:1. The effectors and targets were co-cultured overnight for 16h, before target cell recognition was monitored by measuring release of IFN-γ into the culture supernatant by ELISA.

The first peptide to be analysed was U90 FES. Responses to this epitope were identified in donors HD05, HD30 and HD57. The HLA types of these donors is HD05 HLA-A2, A24, B40(60), B44; HD30 HLA-A2, A32, B7, B40(60) and HD57 HLA-A2, B40(60), B50). Thus, based on this it was likely that this peptide was presented by A2 or B40, which are the shared alleles between these donors. However, as described earlier (section 5.1.3) this peptide had been identified by Gerdmann *et al.,* where they showed that is was presented by HLA-B40 (Gerdmann *et al.,*2013). To confirm the response identified here was similarly presented by B40, FES-specific T cells from donor HD05 were used as effectors with peptide-pulsed autologous, B40-matched or mis-matched PHA blasts as targets. T cells reactive against FES recognised autologous and B40-matched targets matched at HLA-B40(60) with no

significant reactivity detected against mis-matched targets (Figure 5.26). The VEE peptide was also identified here in the same three donors (section 5.1.3), and also mapped by Gerdemann to HLA B40. Experiments were not carried out to confirm this, but it was assumed that the response identified in this thesis was also restricted through HLA-B40.

T cells recognising the U90 peptide PSK were identified in donors HD53. Donor HD53 was homozygous for HLA-A29 and –B44. Thus, PSK peptide-pulsed autologous and A29- or B44-matched PHA blasts were used as targets for polyclonal T cell generated by stimulation of PBMC from HD53 with the 9-mer peptide PSK. T cell recognition of target cells was determined by measuring the release of IFN-γ in to the supernatant by ELISA. T cells reactive against PSK recognised autologous and allogeneic targets matched at HLA-A29 but not HLA-B44 (Figure 5.27), implying that PSK was presented in the context of HLA-A29.

T cells recognising the U90 peptide LNI were identified in donor HD49. Donor HD49 was HLA-A1,-A2, -B8, -B44. Thus, LNI peptide-pulsed autologous and A1-, A2-, B8- or B44-matched PBMC were used as targets this time, to reduce the background. Polyclonal T cells generated by stimulation of PBMC for HD49 with the 9-mer peptide LNI were used as effectors. T cell recognition of target cells was determined by measuring the release of IFN-γ in to the supernatant by ELISA. T cells reactive against LNI recognised autologous and allogeneic targets matched at HLA-A1 but not others (Figure 5.28), implying that LNI was presented in the context of HLA-A1.

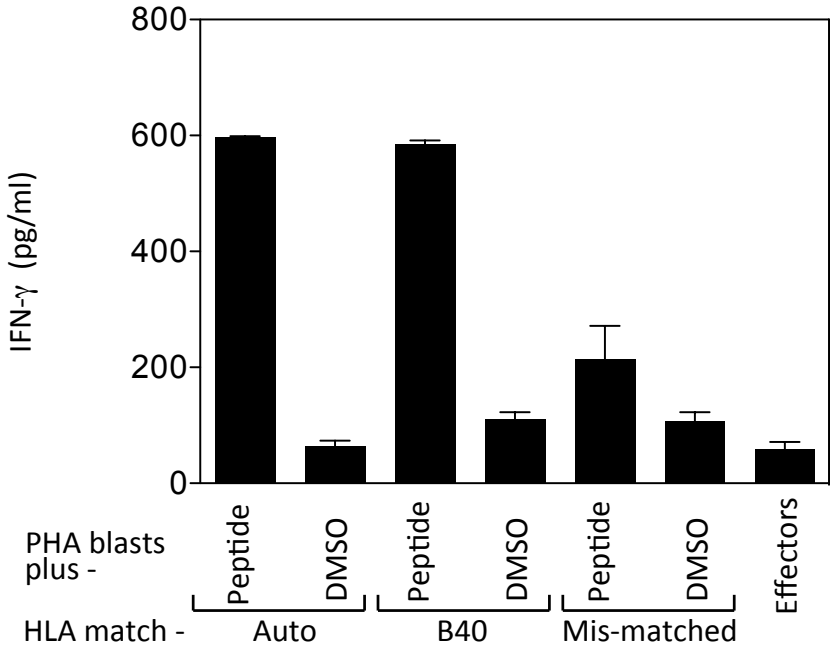


Figure 5.26 Identification (Confirmation) of HLA-B40 as the restriction element for U90 FESLLFPEL (aa 57-65) in donor HD05 [HLA-A2, A24, B40(60), B44]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD05 with the U90 9-mer peptide FESLLFPEL and cultured for 10 days in the presence of IL-2, were tested against autologous, B40-matched or B40-ve (mis-matched) PHA blasts were pulsed with either the FESLLFPEL peptide (2 μg/ml) or DMSO. Polyclonal T cells were used used in T cell assays at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant. T cell effector cells alone were used as negative control.

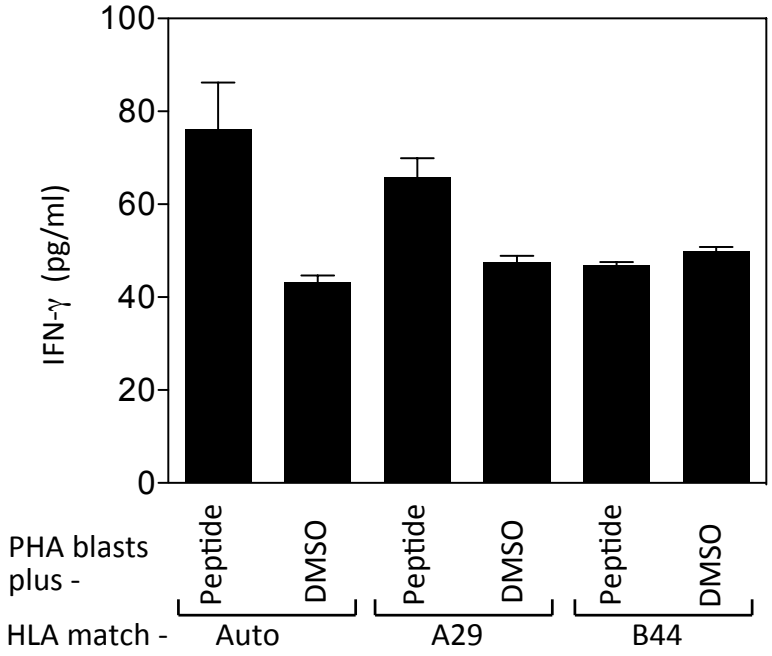


Figure 5.27 HLA restriction of the U90 peptide epitope PSKSKKIKL (aa758–766) maps to HLA-A29 in donor HD53 [HLA-A29, B44]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD53 with the U90 9-mer peptide PSK and cultured for 10 days in the presence of IL- 2, were tested against autologous, A29-matched or B44-matched PHA blasts pulsed with either the PSKSKKIKL peptide (2 μg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant.

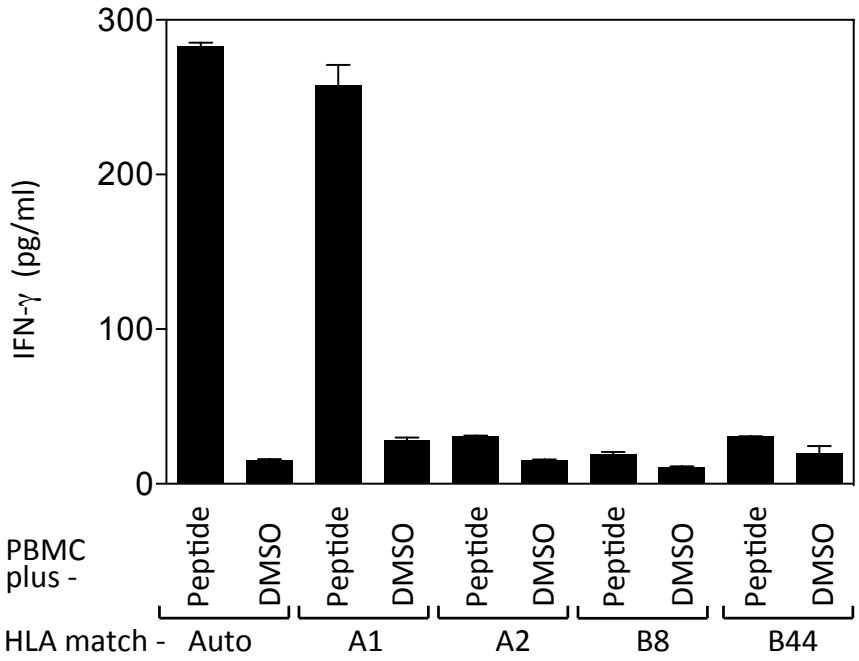


Figure 5.28 HLA restriction of the U90 peptide epitope LNIDPSESI (aa417–425) maps to HLA-A1 in donor HD49 [HLA-A1, A2, B8 ,B44]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD49 with the U11 9-mer peptide LNIDPSESI and cultured for 10 days in the presence of IL-2, were tested against autologous, A1-matched, A2-matched, B8-matched or B44-matched PBMC pulsed with either the LNIDPSESI peptide (2 μg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant.

T cells recognising the U90 peptide NHC were identified in donor HD10. Donor HD10 was HLA-A1, -A2, -B8, -B39. Thus, NHC peptide-pulsed autologous and A1-, A2-, B8- or B39-matched PBMC were used as targets for polyclonal T cells generated by stimulation of PBMC for HD10 with the 9-mer peptide NHC. T cells reactive against NHC recognised autologous and allogeneic targets matched at HLA-B39 but not others (Figure 5.29), implying that NHC was presented in the context of HLA-B39.

T cells recognising the U90 peptides NLI and ITA were identified in donor HD27 (HLA-A1, -A2, -B44) and also predicted to bind to HLA-A2 (see section 5.1.1). To confirm this, firstly NLI peptide-pulsed autologous and A1-, A2- or B44-matched PBMC were used as targets for polyclonal T cells generated by stimulation of PBMC for HD27 with the 9-mer peptide NLI. T cells reactive against NLI recognised autologous and allogeneic targets matched at HLA-A2 but not others (Figure 5.30A), implying that NLI was presented in the context of HLA-A2. In a separate experiment with same donor HD27, the U90 peptide ITA was also shown to be presented in the context of HLA-A2 (Figure 5.30B).

T cells recognising the U11 peptide LKT were identified in donor HD32 (HLA-A1, -A33, -B37, -B58). Thus, LKT peptide-pulsed autologous and A1-, A33-, B37- or B58-matched PBMC were used as targets for polyclonal T cells generated by stimulation of PBMC for HD32 with the 9-mer peptide LKT. T cells reactive against LKT recognised autologous and allogeneic targets matched at HLA-B37 but not others (Figure 5.29), implying that LKT was presented in the context of HLA-B37.

Overall, a number of minimal peptides were identified in U90 and U11, both novel and confirming peptides identified by others during the course of this thesis. HLA restriction elements have been mapped to these peptides. Table 5.1 shows a summary of this data with amino acid coordinates for each peptide.

A number of 15-mer peptides were also identified but for various reasons it was not possible to follow these up further to map the minimal epitopes or identify HLA restriction elements. These may potentially contain novel CD8+ T cell epitopes. This data is summarised in Table 5.2. However, towards the end of the thesis it was possible to generate polyclonal lines to a number of these 15-mers to allow for studies of whether these peptides were presented in HHV6B-infected cells (section 5.4), lending support their identification as containing HHV6B T cells epitopes.

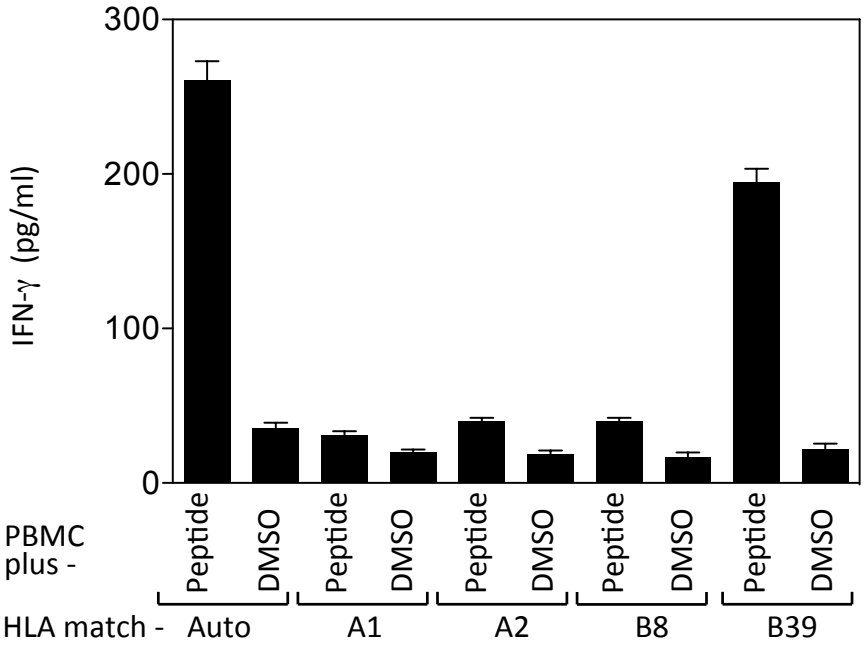


Figure 5.29 HLA restriction of the U90 peptide epitope NHCFINHFV (aa903–911) maps to HLA-B39 in donor HD10 [HLA-A1, A2, B8, B39]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD49 with the U90 9-mer peptide NHCFINHFV and cultured for 10 days in the presence of IL- 2, were tested against autologous, A1-matched, A2-matched, B8-matched or B39-matched PBMC pulsed with either the NHCFINHFV peptide (2 μg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant.

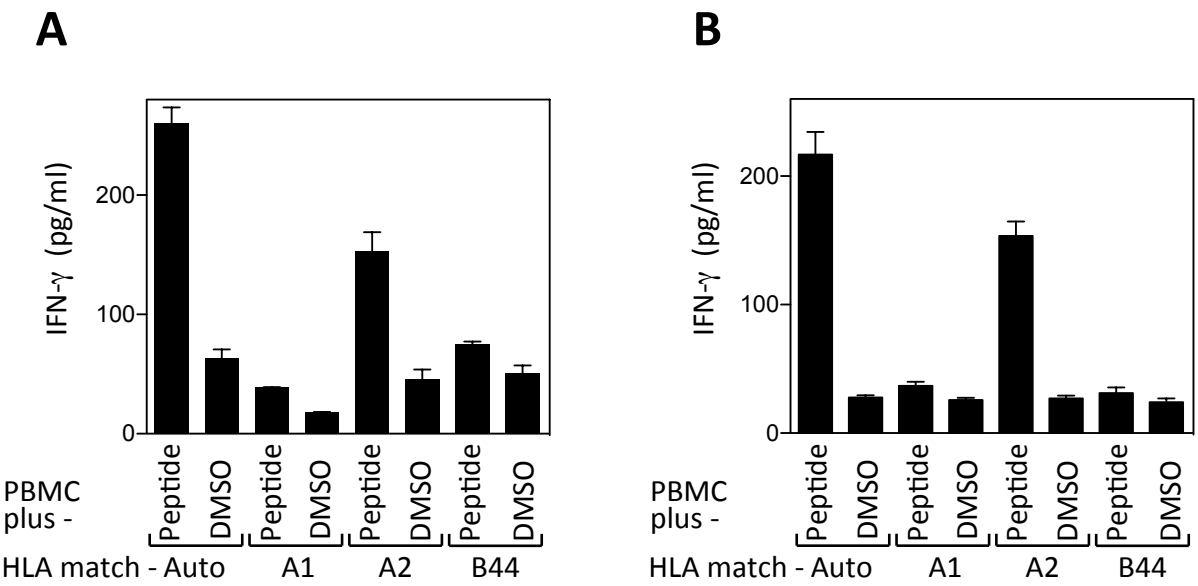


Figure 5.30 HLA restriction of the U90 peptide epitopes NLITAAKNI (aa331-339) and ITAAKNIGI (aa333-341) maps to HLA-A2 in donor HD27 [HLA-A1, A2, B44]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD49 with the U90 9-mer peptides NLITAAKNI (A) or ITAAKNIGI (B), then cultured for 10 days in the presence of IL- 2, were then tested against autologous, A1-matched, A2-matched or B44-matched PBMC pulsed with either the NLITAAKNI (A) or ITAAKNIGI (B) peptides (2 μg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant.

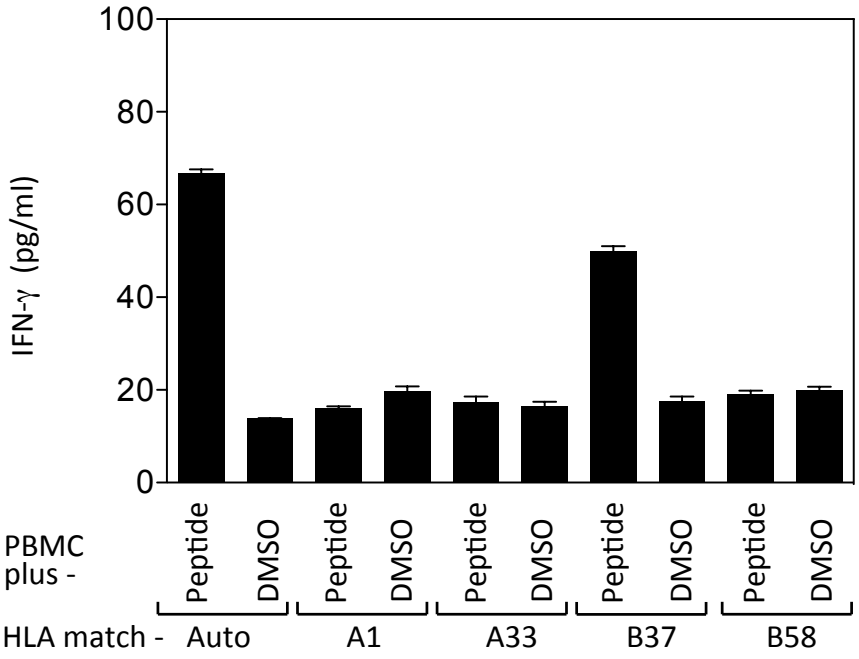


Figure 5.31 HLA restriction of the U11 peptide epitope LKTQRRHKF (aa338–346) maps to HLA-B37 in donor HD32 [HLA-A1, A33, B37, B58]

Polyclonal T cell effectors generated by pulsed PBMC from HLA-matched donor HD32 with the U11 9-mer peptide LKTQRRHKF and cultured for 10 days in the presence of IL- 2, were tested against autologous, A1-matched, A33-matched, B37-matched or B58-matched PBMC pulsed with either the LKTQRRHKF peptide (2 μg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated readings ± SD (errors bars) and expressed as pg/ml IFN-γ released into the supernatant.

Table 5.1 CD8 T cells 9-mer epitopes identified in HHV6B U90 and U11

HHV6B antigen	aa position	aa sequence	HLA
U90			
	39-47	VEESIKEIL	B40(60)
	57-65	FESLLFPEL	B40(60)
	331-339	NLITAAKNI	A2
	333-341	ITAAKNIGI	A2
	417-425	LNIDPSESI	A1
	758-766	PSKSKKIKL	A29
	903-911	NHCFINHFV	B39
U11			
	238-246	FNAVYSQRV	NT*
	338-346	LKTQRRHKF	B37
	413-421	GILDFGVKL	A2

*not tested

Table 5.2 CD8 T cells 15-mer potential epitopes identified in HHV6B U90 and U11

HHV6B antigen	amino acid position	15-mer sequence	HLA
U90			
	33-47	YHPDPVVEESIKEIL*	B40(60)
	53-67	CDVSFESLLFPELEA*	B40(60)
	209-223	ASESRDLLMDLKANM	NT**
	324-338	IHINCKNLITAAKNI*	A2
	329-343	CKNLITAAKNIGIAV*	A2
	413-427	SICDLNIDPSESILL*	A1
	453-467	NVDYLKYMEVQSPTD	NT
	693-707	CDSTVRTNNIHMNNT	NT
	766-780	KPSKSKKIKLDRLPE*	A29
	849-863	IPIRSMPGTHDIRNK	B44
	869-883	HWLWFMRKTHKVDNC	NT
	874-888	FMRKTHKVDNCVIHS	NT
	901-915	EANHCFINHFVPIKT*	B39
	905-919	CFINHFVPIKTDDEE	B44
	921-935	EKENVSYTYSKIQDS	NT
	1013-1027	GNNSPILNIINDTTC	NT
U11			
	29-43	ENLENVDLREHPYVT	NT
	65-79	ILWLMYHYVLSKRKP	NT
	93-107	NVVNEYLKSKGLNKG	NT
	97-111	EYLKSKGLNKGNFEN	NT
	193-207	INKLVYLGKLFVTLN	NT
	233-247	RKNENFNAVYSQRVL*	NT
	237-251	NFNAVYSQRVLQTPL	NT
	273-287	SLESYSASKAFSVPE	NT
	277-291	YSASKAFSVPENGPH	NT
	309-323	LPSISIDTKRPSADL	NT
	337-351	PLKTQRRHKFPESDS*	B37
	349-363	SDSVDNAGGKILIKK	NT
	353-367	DNAGGKILIKKETLG	NT
	413-427	GILDFGVKLPAEAQS*	A2
	433-447	DLLQDKTSIRSPSSG	NT
	749-763	ADLSRDLDSVSFKDA	NT

* 9-mer has been mapped, **not tested

5.4 T cell recognition of HHV6B infected cells

To determine if T cells recognising the newly identified HHV6B peptide epitopes could recognise HHV6B infected cells, autologous PHA blasts were infected with HHV6B as described in the material and methods (section 2.15), before being used as targets for epitope specific polyclonal T cells for each newly identified epitope. In addition, testing the recognition of HHV6B infected cells for the 9-mer peptides (U90; VVEESIKEI, FESLLFPEL, NLITAAKNI, ITAAKNIGI, LNIDPSESI, PSKSKKIKL, NHCFINHFV and U11; LKTQRRHKF), this was also carried out for 15-mer peptides (U90; ASESRDLLMDLKANM, CDSTVRTNNIHMNNT, IPIRSMPGTHDIRNK and CFINHFVPIKTDDEE and U11; INKLVYLGKLFVTLN, SLESYSASKAFSVPE, YSASKAFSVPENGPH and DNAGGKILIKKETLG). T cells specific for each peptide to be tested were generated by stimulating PBMC with each individual peptide, and cultures were maintained for 10 days at 37°C, with IL2 being added to cultures from day 3. Polyclonal cultures were then used as effectors in virus recognition assays. Target cells incubated with effectors at an Effector-to-Target (E:T) ratio of 10:1 (in some experiments more than one E:T ratio was used). The effectors and targets were co-cultured overnight for 16h, before target cell recognition was monitored by measuring release of IFN-γ into the culture supernatant by ELISA.

The first peptide to be analysed was PSK, which maps to U90 aa758-766. To confirm this, polyclonal PSK-specific T cell effectors generated by pulsed PBMC from HLA-matched donor HD53 with the U90 9-mer peptide PSK and cultured for 10 days in the presence of IL- 2, were tested against autologous PHA blasts infected with HHV6B or mock infected. Or as controls PHA blasts pulsed with either the PSK peptide or DMSO. Polyclonal T cells were used at three E:T ratio. Thus, HHV6B

infected- or mock infected-autologous PHA blasts were used as targets for polyclonal T cells generated by stimulation of PBMC for HD53 with the 9-mer peptide PSK. HHV6B infection was confirmed by immunofluorescence assay (IFA) analyses (Figure 5.32B) T cell recognition of target cells was determined by measuring the release of IFN-γ in to the supernatant by ELISA. T cells reactive against PSK recognised autologous PHA blasts infected with HHV6B but not mock infected (Figure 5.32B), implying that T cells reactive against PSK was able to kill HHV6B infected PHA blasts.

Similar experiments were conducted for another U90 9-mer peptide identified and associated with HLA-B40 aa57-65 FESLLFPEL and T cells expanded were able to kill HHV6B infected PHA blasts (Figure 5.33). Additionally, similar experiments were conducted for other U90 peptides identified and T cells expanded were able to kill HHV6B infected PHA blasts (Figure 5.34). Also, similar experiments were conducted for another U11 9-mer peptide identified and associated with HLA-B37 aa338–346 LKTQRRHKF and T cells expanded were able to kill HHV6B infected PHA blasts (Figure 5.35A). Furthermore, similar experiments were conducted for other U11 15-mer peptides identified and T cells expanded were able to kill HHV6B infected PHA blasts (Figure 5.35).

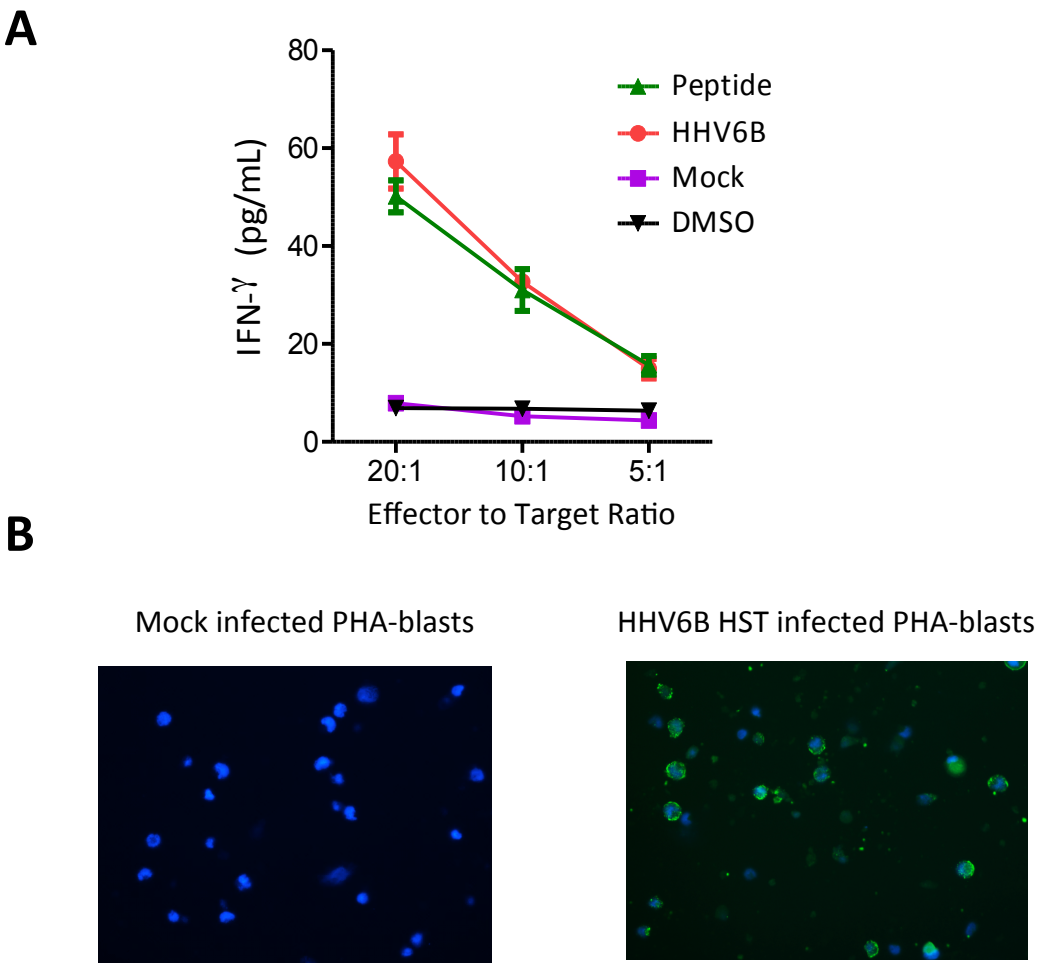


Figure 5.32 Recognition of HHV6B infected cells by U90 PSK-specific effector T cells

(A) Polyclonal PSK-specific T cell effectors generated by pulsed PBMC from HLA-matched donor HD53 with the U90 9-mer peptide PSK and cultured for 10 days in the presence of IL-2, were tested against autologous PHA blasts infected with HHV6B strain HST or mock infected. Or as controls PHA blasts pulsed with either the PSK peptide (2 µg/ml) or DMSO. Polyclonal T cells were used at the indicated E:T ratio. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated ± SD (errors bars) readings and expressed as pg/ml IFN-γ released into the supernatant. (B) To confirm efficient viral infection of target cells, HHV6B-infected and mock-infected cells used in the assay in (A) were stained with mouse-anti–human HHV6B mAb (monoclonal antibody MAB8535, clone C3108-003; Chemicon). Immunofluorescence images where taken with a Nikon fluorescence microscope, magnification 400x.

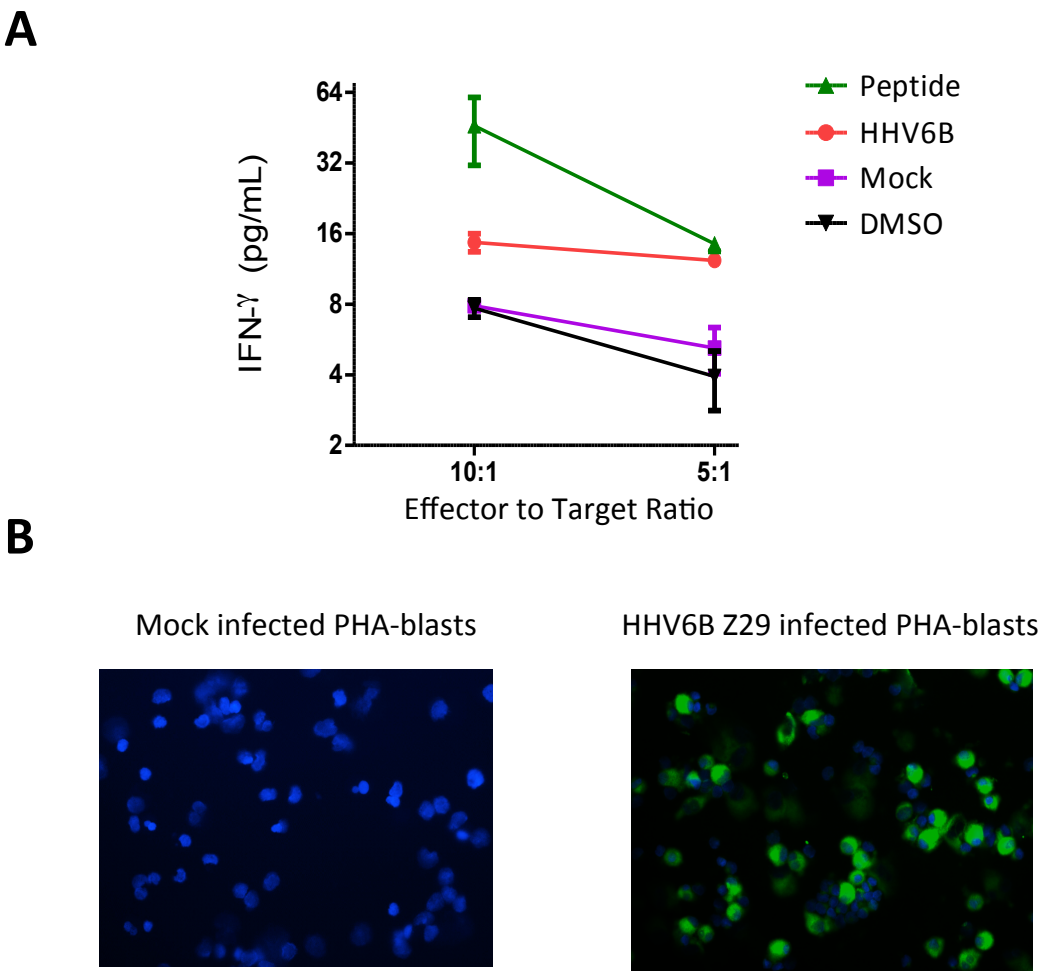


Figure 5.33 Recognition of HHV6B infected cells by U90 FES-specific effector T cells

Polyclonal FES-specific T cell effectors generated by pulsed PBMC from HLA-matched donor HD57 with the U90 9mer peptide FES and cultured for 10 days in the presence of IL-2, were tested against autologous PHA blasts infected with HHV6B strain Z29 or mock infected. Or as controls PHA blasts pulsed with either the FES peptide (2 µg/ml) or DMSO. Polyclonal T cells were used at the indicated E:T ratio. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated ± SD (errors bars) readings and expressed as pg/ml IFN-γ released into the supernatant. (B) To confirm efficient viral infection of target cells, HHV6B-infected and mock-infected cells used in the assay in (A) were stained with mouse-anti–human HHV6B mAb (monoclonal antibody MAB8535, clone C3108-003; Chemicon). Immunofluorescence images where taken with a Nikon fluorescence microscope, magnification 400x.

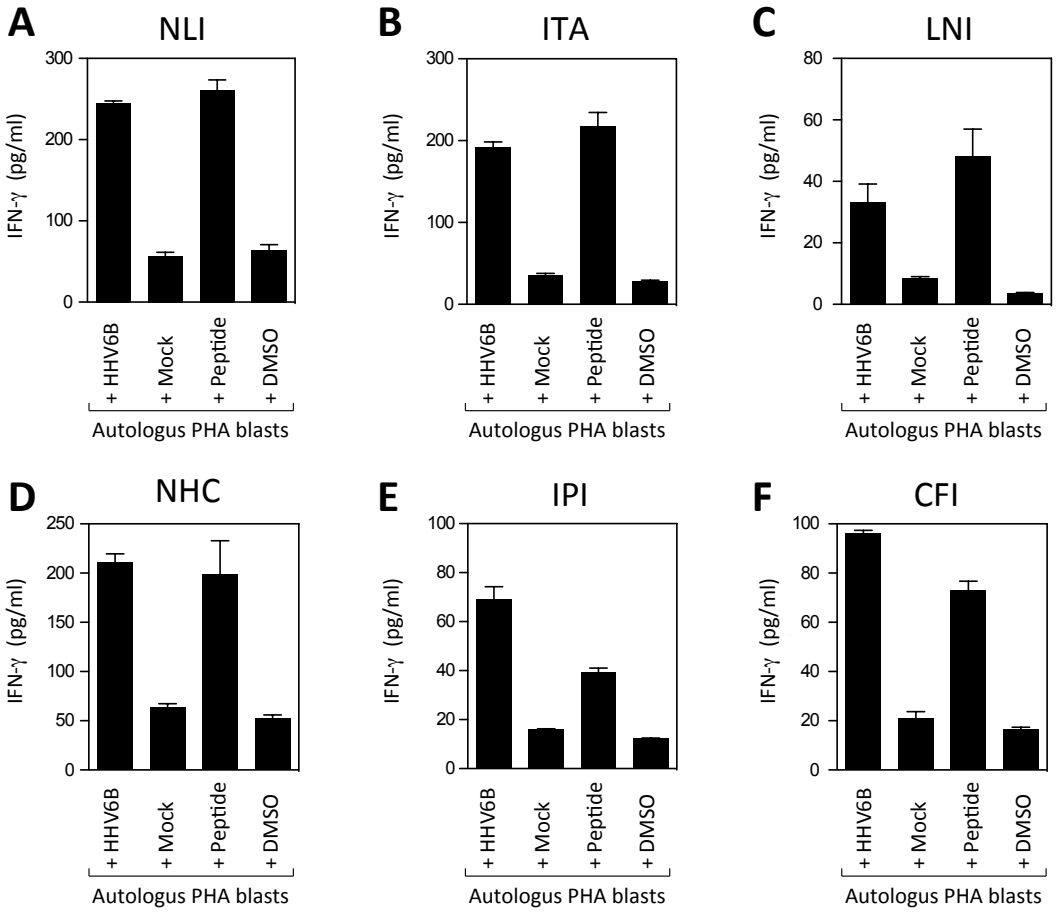


Figure 5.34 Recognition of HHV6B infected cells by U90 peptide-specific effector T cells

Polyclonal peptide-specific T cell effectors generated by pulsed PBMC from HLA-matched donor HD27 with the U90 9-mer peptide NLI (A) and ITA (B), and PBMC from HLA-matched donor HD49 with the U90 9-mer peptide LNI (C), and PBMC from HLA-matched donor HD10 with the U90 9-mer peptide NHC (D), and PBMC from HLA-matched donor HD53 with the U90 15-mer peptide IPI (F) and CFI (E). Then cells cultured for 10 days in the presence of IL-2, were tested against autologous PHA blasts infected with HHV6B strain Z29 or mock infected, or as controls PHA blasts pulsed with either the FES peptide (2 µg/ml) or DMSO. Polyclonal T cells were used at an E:T ratio of 10:1. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated ± SD (errors bars) readings and expressed as pg/ml IFN-γ released into the supernatant.

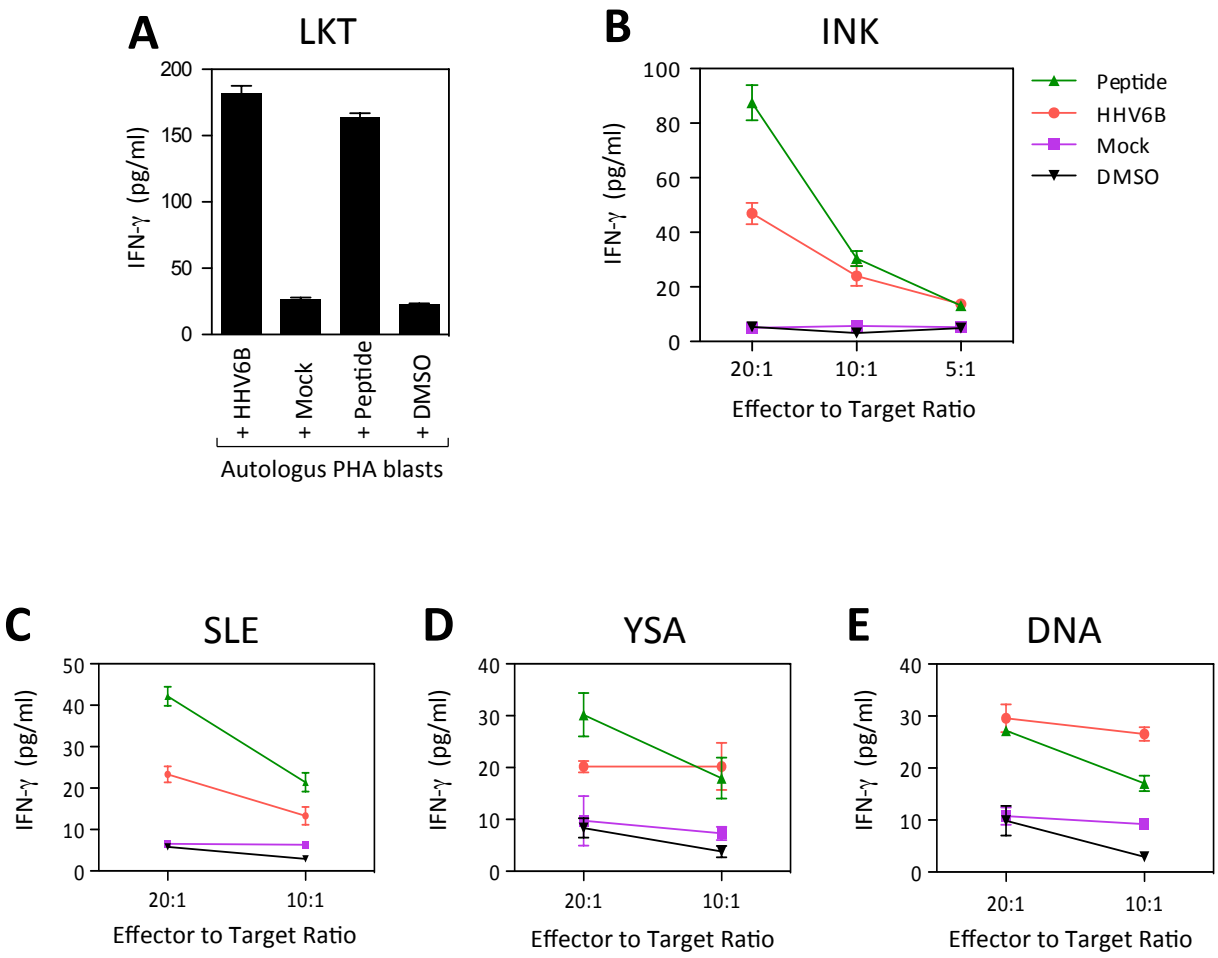


Figure 5.35 Recognition of HHV6B infected cells by U11 peptide-specific effector T cells

Polyclonal peptide-specific T cell effectors generated by pulsed PBMC from HLA-matched donor HD32 with the U11 9-mer peptide LKT (A), and PBMC from HLA-matched donor HD24 with the U11 15-mer peptide INK (B), SLE (C), YSA (D) and DNA (E). Then cells cultured for 10 days in the presence of IL- 2, were tested against autologous PHA blasts infected with HHV6B strain Z29 or mock infected, or as controls PHA blasts pulsed with either the LKT peptide (2 µg/ml) or DMSO. Polyclonal T cells were used for LKT (A) at an E:T ratio of 10:1 and were used for the 15-mers at the indicated E:T ratio. T cell recognition of target cells was determined by measuring release of IFN-γ in to the supernatant by ELISA. Results are the mean of triplicated ± SD (errors bars) readings and expressed as pg/ml IFN-γ released into the supernatant.

Table 5.3 Summary of CD8 T cells peptides identified in HHV6B U90 and U11

HHV6B antigen	15-mer sequence	aa position	9-mer sequence	HHV6 Recognition
U90				
	YHPDPVVEESIKEIL	33-47	VEESIKEIL	Yes
	CDVSFESLLFPELEA	53-67	FESLLFPEL	Yes
	ASESRDLLMDLKANM	209-223	NT*	Yes
	IHINCKNLITAAKNI	325-339	NLITAAKNI	Yes
	CKNLITAAKNIGIAV	329-343	ITAAKNIGI	Yes
	SICDLNIDPSESILL	413-427	LNIDPSESI	Yes
	NVDYLKYMEVQSPTD	453-467	NT	NT
	CDSTVRTNNIHMNNT	693-707	NT	Yes
	KPSKSKKIKLDRLPE	757-771	PSKSKKIKL	Yes
	IPIRSMPGTHDIRNK	849-863	NT	Yes
	HWLWFMRKTHKVDNC	869-883	NT	NT
	FMRKTHKVDNCVIHS	874-888	NT	NT
	EANHCFINHFVPIKT	901-915	NHCFINHFV	Yes
	CFINHFVPIKTDDEE	905-919	NT	Yes
	EKENVSYTYSKIQDS	921-935	NT	NT
	GNNSPILNIINDTTC	1013-1027	NT	NT
U11				
	ENLENVDLREHPYVT	29-43	NT	NT
	ILWLMYHYVLSKRKP	65-79	NT	NT
	NVVNEYLKSKGLNKG	93-107	NT	NT
	EYLKSKGLNKGNFEN	97-111	NT	NT
	INKLVYLGKLFVTLN	193-207	NT	Yes
	RKNENFNAVYSQRVL	233-247	FNAVYSQRV	NT
	NFNAVYSQRVLQTPL	237-251	NT	NT
	SLESYSASKAFSVPE	273-287	NT	Yes
	YSASKAFSVPENGPH	277-291	NT	Yes
	LPSISIDTKRPSADL	309-323	NT	NT
	PLKTQRRHKFPESDS	337-351	LKTQRRHKF	Yes
	SDSVDNAGGKILIKK	349-363	NT	NT
	DNAGGKILIKKETLG	353-367	NT	Yes
	GILDFGVKLPAEAQS	413-427	GILDFGVKL	NT
	DLLQDKTSIRSPSSG	433-447	NT	NT
	ADLSRDLDSVSFKDA	749-763	NT	NT

*not tested

5.5 Summary

In conclusion, this chapter has presented the Identification of HLA Class I restricted antigenic peptides in HHV6B U11 and U90. Then, the chapter went on to characterise these responses in more detail, by mapping the minimal antigenic peptides, identifying the HLA restriction elements and determining whether these T cells were capable of killing HHV6B-infected cells. This is important information to fully characterise the CD8+ T cells response to HHV6B, to support any future application of such T cell therapy against HHV6B driven disease.

Chapter 6

6. General discussion and conclusions

6.1 Discussions

Viruses are major causes of morbidity and mortality after bone marrow transplant (BMT), for example the viruses CMV, EBV, AdV, BK, HHV6, HHV7 and RSV. HHV6B reactivation can cause serious complications in immunocompromised patients such as BMT recipients. These reactivations are normally controlled by the immune system in immunocompetent subjects; but immunocompromised patients (such as in BMT recipients) may be unable to contain them leading to life threatening conditions; like, graft versus host disease (GVHD), delayed engraftment, encephalitis and pneumonitis (De Bolle *et al.*, 2005a). Prevention of post-transplant viral reactivation is very difficult. Finding a way to control these infections are becoming more and more important considering the increasing number of transplantations in recent years (Zerr *et al.*, 2001; Zerr *et al.*, 2002; Rapaport *et al.*, 2002; Zerr *et al.*, 2005; Karlsson *et al.*, 2007; De Pagter *et al.*, 2008).

When this project was started in 2010, very little information was available regarding the T cell response to HHV6. The immunogenic antigens, potential antigenic peptides, HLA restricting alleles and strength of responses were unknown. Based on the well-characterised cellular immune response to other human herpes viruses (particularly HCMV and EBV), we hypothesised that HHV6 will be immunogenic for T cells. In this project the T cell responses to HHV6 have been characterised in a panel of healthy donors by investigating; direct *ex-vivo* detection of T cell responses to HHV6, *in-vitro* expansion and analyses of T cell responses to HHV6B antigens (U11, U39, U54 and U90), and identification and characterisation of CD8+ T cell responses

to HHV6B antigens U11 and U90. The findings contribute to the expansion of existing information regarding the immune response to HHV6 and are relevant to exploration of the potential of developing adoptive T cell therapy for malignancies linked to HHV6 proliferation.

Adoptive T cell immunotherapy is a promising therapeutic approach for the treatment of diseases associated with reactivation of herpes viruses in immunosuppressed patients. At present, adoptive T cell immunotherapy can successfully prevent and treat fatal infections in immunocompromised individuals. However, benefits of these therapies have been restricted to CMV, EBV and adenovirus (Leen *et al.*, 2006; Karlsson *et al.*, 2007; Leen *et al.*, 2009; Barker *et al.*, 2010). Adoptive immunotherapy may be extended to HHV6 to develop BMT engraftment and control recipients' viral reactivation. Recent studies have shown an HHV6 specific T cell immune response (Nastke *et al.*, 2012; Martin *et al.*, 2012) directed against some viral proteins (U11, U14, U54, U90) in association with HLA-A2, HLA-A3, HLA-B7 or HLA-B40 antigens (Martin *et al.*, 2012; Gerdemann *et al.*, 2013; Iampietro *et al.*, 2014). Identification of additional viral targets (epitopes) presented in association with other HLA class I alleles is therefore needed.

Using synthetic viral peptides to stimulate T cells and evaluating their ability to produce IFN-γ, studies have identified a number of HHV6 antigens recognised by T cell clones in peripheral blood of healthy donors (Nastke *et al.*, 2012; Martin *et al.*, 2012; Gerdemann *et al.*, 2013; Iampietro *et al.*, 2014).

Using algorithmic epitope peptides prediction program (SYFPEITHI) Nastke et al evaluated a 17-mer peptide with a very high affinity for binding to major

histocompatibility complex (HLA) class II (HLA -II) DR1. Due to the high homology between the different HLA-II, peptides capable of binding to HLA -DR1 may also bind to other HLA-II molecules. Screening was also done with a library of peptides derived from the genome of HHV6B translated proteins and another peptide library from six virion proteins that have been established as abundant in cells infected by proteomics. Eleven peptides were found to activate the CD4+ T cell response in PBMC from different healthy donors. Only two of these peptides were part of the peptide library from the complete genome while the other nine belonged to the library of peptides of virion proteins: five mapped to the capsid protein U57, two to tegument protein U11 and U14, and two to glycoproteins U38 and U48 (Nastke *et al.*, 2012). These results confirmed previous studies that determined that the capsid proteins and tegument could activate T cell immune response (Wang *et al.*, 1999; Tejada-Simon *et al.*, 2002).

Martin *et al* evaluated and identified the immune response specific to CD8+ T cells in HLA-A2+ donors, predicted by computational prediction program as theoretically strong for U11, U54 and U90 proteins. Lines and clones have been shown to recognise virus-infected target cells and CD8 + T cells were able to react strongly against CD4+ T cells infected with HHV6B, producing antiviral cytokines and eliminating infected cells (Martin *et al.*, 2012).

Gerdemann *et al.* focused their research on five HHV6 proteins homologous to immunodominant antigens identified in HCMV, and T cell responses mapped to HHV6B antigens U11, U14, U54, U71 and U90. Also, they have mapped three B40-restricted epitopes to U90 and U14 (Gerdemann *et al.*, 2013).

A recent study by Iampietro *et al* identified six epitopes derived from U90 proteins in the immune response of specific CD8+ T cells restricted to HLA-I alleles, HLA-A2, HLA-A3, and HLA-B7. These epitopes were functionally tested for their ability to induce $CD8^+$ T cell expansion and kill HHV6-infected autologous cells (Iampietro *et al.*, 2014).

Initially, in Chapter 3, four HHV6B antigens U11, U39, U54 and U90, predicted to be immunogenic based on their CMV homologues, were focused upon. PBMC from 30 healthy donors were stimulated with pepmixes corresponding to these four antigens. Whole antigen pepmixes were used to stimulate PBMC. T cell responses were analysed by intracellular cytokine staining (ICS) after overnight stimulation. CD8+ responses were observed to U90 and U54 respectively in a number of donors, but in comparison to other control antigens, these responses were significantly lower in magnitude and frequency (0.02-0.2%). These results were consistent with the findings of other studies reporting less than 0.1% of total PBMC responding to these antigens (Martin *et al.*, 2012; Gerdemann *et al.*, 2013).

However it may be that other untested HHV6B antigens may be more immunogenic, or that HHV6B may be less immuno-stimulatory than HCMV. Therefore, to maximise the ability to detect HHV6B antigen-specific T cells and determine the antigenicity of the 4 antigens being studied, a short-term *in-vitro* reactivation protocol was utilised to generate a polyclonal culture enriched with HHV6B-specific T cells prior to analysis by ELISpot.

This frequency is increased in transplant patients, showing a good response with HLA tetramers for HHV6 specific peptides (Gerdemann *et al.*, 2013). Stimulation of non-

enriched PBMC, even using the tetramers, is unable to produce a strong response. For this reason, enrichment of positively stained cells by tetramers was employed to establish an association between clones of CD4+ T lymphocytes and certain epitopes (Vollers and Stern, 2008). An additional limitation of this technique is that HLA tetramers are only available for a limited number of HLA alleles and furthermore are only able to be used in HLA matched donors. The *ex-vivo* study of T cell immune response to HHV6 in PBMC is limited due to the low frequency of specific T cell antigens. For this reason, researches focussed on *in-vitro* expansion of T cells for easier identification of epitopes.

The next approach (in Chapter 4) included an *in-vitro* stimulation step, with successful expansion of the HHV6B (U11, U39, U54 and U90) antigen-specific CD8+ T cells in 25 healthy donors. Short-term *in-vitro* reactivations of PBMC were performed with HHV-6B pepmixes. Subsequently, HHV6B antigen-specific CD8+ T cell responses were analysed by IFN-γ ELISpot assay; showing 88% (22/25), 84% (21/25), 76% (19/22) and 72% (18/25) of the donors responding to U54, U90, U11 and U39 antigens respectively. The responses in this study were a little lower than the findings of other studies, showing all of the donors responding against U11, U54 and U90 (Gerdemann *et al.*, 2013).

This analysis had not defined the component of the polyclonal culture of HHV6B-stimulated T cells in terms of whether this was mainly CD4+ or CD8+ T cells, or an equal contribution from both or if they are similar to the normal reference range for peripheral blood lymphocytes, rather than also determining antigen specificity. The result of the frequencies of CD4+ and CD8+ T cells in 10-day polyclonal cultures,

stimulated with each HHV6B pepmix, were measured by flow cytometry. These expanded polyclonal cultures of HHV6B-stimulated cells consist of CD3+ T cells (mean 86.9% ± 6%), with a majority of helper (CD4+) T cells (66.3% ± 4.5%) and a minority of CD8+ T cells (25.9% ± 3.2%). This is similar to the reference values of CD3+ (61 to 85%), CD4+ (28 to 58%), and CD8+ (19 to 48%) (Reichert *et al.*, 1991).

In-vitro stimulation steps in the presence of IL-2 can successfully expand HHV6B antigen-specific T cells. T cell responses were detected against all HHV6B antigens tested, U11, U39, U54 and U90. These expanded polyclonal cultures of HHV6B-specific T cells consist of CD3+ T cells, with a majority of CD4+ T cells and a minority of CD8+ T cells. Overall all the 25 donors responded to at least one of the HHV6B antigens. In general these responses were of high magnitude, in comparison to *ex-vivo* responses. Therefore, the next stage was to characterise these responses in more detail, by mapping the minimal antigenic peptides, identifying the HLA restriction elements and determining whether these T cells were capable of killing HHV6B-infected cells.

Subsequently, in Chapter 5, the breadth of epitope specificity within U90 and U11 was screened for 9 healthy donors using overlapping peptides. The responses to U11 and U90 peptides libraries were used to map CD8+ T cell restricted epitopes with characterisation and identification of HLA restricting elements and cytokine profiles. We have identified 10 CD8+ T cells specific (9-mer) epitopes within these antigens. Seven of them were within U90 antigens and three of them were within U11 antigens. Allelic association of the U90 epitopes were; VEESIKEIL - B40 (60), FESLLFPEL - B40 (60), NLITAAKNI - A2, ITAAKNIGI - A2, LNIDPSESI - A1, PSKSKKIKL -

A29, NHCFINHFV - B39. Allelic association of the 2 U11 epitopes were LKTQRRHKF - B37 and GILDFGVKL – A2; the HLA association to FNAVYSQRV was not identified. CD8+ T cell populations specific to some of these epitopes were also expanded, which were able to kill HHV6B infected cells. Characterisation and identification of MHC class I restriction of these U90 and U11 epitopes; and their ability to induce antigen-specific CD8+ T cells that are able to recognise HHV6+ve cells would be helpful to possible future development of adoptive immune therapy.

Researchers have started to explore and identify HHV6 specific CD8+ T cell responses in recent times. The specific immunodominant epitopes in HHV6B to CD8+ T cells was first reported in 2012, showing three epitopes within U11 and 2 epitopes within U54 (Martin *et al.*, 2012). Later in 2013, researchers were able to characterise three HHV6 epitopes within U14 (1 epitope) and U90 (2 epitopes) specific for CD8+ T cells, all restricted to HLA-B40 (Gerdemann *et al.*, 2013). In addition, their study demonstrated that these epitopes were polyfunctional, producing IFN-γ, TNF-α and granzyme-B and exerting cytotoxicity to HHV6B infected cells (Gerdemann *et al.*, 2013). In this study seven epitopes of U90 were characterised; two of them (restricted to HLA-B40) were identical to their report and the other five restricted to A2, A1, A29 and B39. Three epitopes were also identified within the U11 antigen; one of them was restricted to A2, which was identical with reports of Martin et al. One of the other two epitopes was restricted to B37 and the third was not detectable due to inadequate cell numbers. Very recently, in 2014, Iampietro *et al* reported four epitopes within U90, none of them matching with the present findings (Iampietro *et al.*, 2014).

This study had a few limitations that need to be considered before drawing definitive conclusions. First of all, it was not possible to ascertain the actual timing when donors became infected with HHV6 and also it was not possible to test the HHV6 seropositivity status. Secondly, cross-reaction between the epitopes of HHV6A and HHV6B could not be excluded. Although HHV6A and HHV6B share 90% homology (Dominguez *et al.*, 1999), the pathophysiology following primary infection or reactivation of these two strains are different. Because of their high homology, less than 10% of the T cell clones for the same epitope are specific to a single species of these two (Yasukawa *et al.*, 1993). A recent study reported cross-reactive responses in one of the HLA-A2 restricted epitopes (Iampietro *et al.*, 2014). The sequences of the epitopes identified in HHV6B in this study were different than those in HHV6A (Shown in Table 6.1).

Table 6.1: Peptide sequences and amino acid coordinates of CD8+ T cell epitopes identified in U90 and U11

HHV-6B antigen	HHV-6B	HHV-6A	HLA restriction element	Recognition of infected targets
U90	VEESIKEIL	**M**EESIKEIL	B40 (60)	Yes
	FESLLFPEL	F**DD**L**II**P**G**L	B40 (60)	Yes
	NLITAAKNI	NLITAAKN**L**	A2	Yes
	ITAAKNIGI	ITAAKN**L**G**N**	A2	Yes
	LNIDPSESI	**G**N**T**DPS**D**S**M**	A1	Yes
	PSKSKKIKL	**SP**K**N**KKIK**T**	A29	Yes
	NHCFINHFV	**K**HCF**M**NHFV	B39	Yes
U11	LKTQRRHKF	L**RM**QR**QP**KF	B37	Yes
	GILDFGVKL	GILDF**N**VK**F**	A2	Yes
	FNAVYSQRV	FN**I**VYS**H**RV	nt	nt

Three putative novel CD8 T cell epitopes were identified in U90, named PSK, NHC and LNI. These epitopes were predicted to be restricted via HLA-A29, B39 and A1 respectively. These HLA alleles predicted to present the novel HHV6B T cell peptides are present in the caucasian population at average frequencies for A1 14.07%, A29 3.01% and B39 2.03% (Marsh *et al.*, 2000), highlighting their therapeutic relevance. The B39-restricted NHC epitope sequence conforms to the motif of peptides known to bind to HLA-B*3901, that have a highly conserved His at position 2, an aliphatic anchor at position 9. The LNI peptide, predicted to be restricted via HLA-A1, gave a weak response in comparison to the original 15-mer, and as HLA-A1 tends to have acidic residues at position 3, rather than position 4 for this peptide it is possible that the real epitope begins NID and may be a 10-mer (Rammensee *et al.*, 1999).

Moreover, with regard to the potential impact of the chromosomal insertion of HHV6, it is unclear whether CIHHV6 makes any difference in the immune response to HHV6. For example, does the high virus load in subjects with CIHHV6 affect specific T cell responses to HHV6? Understanding the ability of the adaptive immune response to HHV6 in relation to CIHHV6 would be additional indirect evidence that the integrated HHV6 genome is perhaps functionally active and is recognised as 'non-self' by the carrying individual. However, CIHHV6 long term persistence (latency) allows HHV6 to reduce detection by immune cells (Kaufer and Flamand., 2014). So, it would be of interest to investigate any fluctuations and frequencies of HHV6 specific T cells, which might protect against the danger of viral reactivation and, perhaps, against linked clinical manifestations, either in immunocompromised or immunocompetent CIHHV6 individuals.

Future studies should be undertaken for more detailed characterisation of the immune response to HHV6, extending the number of antigens that have been looked into, aiming to identify and characterise more epitopes; and to search for CD4+ T cell specific epitopes. In these *in-vitro* studies only IFN-γ producing cells have been analysed. TNF-α is the second major cytokine produced against viruses. Only one published study has shown some HHV6 antigens producing TNF-α responses (Gerdemann *et al.*, 2013). Attempts to explore the epitopes inducing TNF-α responses would be valuable. Future researches should also be targeted for further phenotypic analysis and identification of MHC class II restricted epitopes.

6.2 Conclusions

The prevalence of HHV6 infection is very high and over 90% of the world's population are at risk of viral reactivation. With the increasing number of tissue transplantation procedures being performed in recent times, HHV6 is considered as one of the major threats for post-transplant infections. Although, more than 25 years have passed since the discovery of HHV6, a lot of areas regarding the potential therapeutic targets remain elusive.

The results of this study contribute to expanding the existing information on the interaction of human herpes virus 6 (HHV6) and the human immune system. This study has identified and characterised immunodominant epitopes of HHV6B U90 and U11 proteins, which were able to stimulate and expand CD8+ T cell responses, capable of killing HHV6 infected cells. This information would be valuable to explore the potential to develop adoptive T cell therapy for malignancies linked to HHV6 reactivation.

Chapter 7

7. References

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Chapter 8

8. Appendix

Table 8.1 List of donors with HLA typing and CMV seropositivity

Donor ID	HLA-A	HLA-B	CMV Status
HD01	2,3	8,44	+
HD02	2,32	27,44	+
HD03			+
HD04	3,29	44	+
HD05	2, 24	44, 60	+
HD06	3	7,51	+
HD07	3, 25	8,44	+
HD08	3,66	7,27	+
HD09	2,11	18,44	+
HD10	1, 2	8, 39	-
HD11			+
HD12			+
HD13			-
HD14			+
HD15	1, 2	7, 44	+
HD16	1,30	13,37	+
HD17	1,3	8,27	-
HD18	1, 24	8, 62	-
HD19			-
HD20	1,3	7,8	-
HD21	24,33	44,55	+
HD22	1,2	8,62	+
HD23			-
HD24	29,74	35,50	+
HD25	2	8, 62	+
HD26			+
HD27	1, 2	44	-
HD28	29,68	7,44	+
HD29			-
HD30	2, 32	7, 60	+
HD31	1, 2	8, 57	+
HD32	1,33	37,58	+
HD33	11,29	39,44	+
HD34	2,30	13,44	-
HD35			-
HD36			-
HD37	2,3	7,62	+
HD38			-
HD39	2,3	7,44	-
HD40	2,26	38,44	-
HD41	1	37,57	+
HD42			-

HD43			-
HD44	1		+
HD45			+
HD46			+
HD47			+
HD48			+
HD49	1,2	8,44	-
HD50			+
HD51	3, 26	7, 64	+
HD52			+
HD53	29	44	-
HD54			+
HD55	2	7	+
HD56	2, 30	13,62	-
HD57	2	50,60	-
HD58			+
HD59	1,29	8,45	-
HD60	2,25	44,58	-

Table 8.2 Total data for CD8 T cell responses to HCMV antigens by intracellular cytokine staining for IFN-γ∗

Donor	IE1	IE2	pp65	gB	Neg**	Pos***
HD01	0.28	0.02	0.12	0.02	0.01	2.09
HD02	0.07	0.10	0.35	0.12	0.02	4.03
HD03	1.06	0.03	0.11	0.05	0.03	11.18
HD04	0.02	0.03	0.01	0.02	0.02	1.26
HD05	1.54	0.02	0.83	0.03	0.03	5.56
HD06	0.02	0.01	0.03	0.04	0.02	0.05
HD07	0.07	0.04	0.05	0.07	0.04	0.14
HD08	0.15	0.02	0.11	0.20	0.05	0.30
HD09	8.74	0.01	0.16	0.04	0.02	1.27
HD10	NT	NT	NT	NT	NT	NT
HD11	1.03	0.02	0.58	0.06	0.02	3.54
HD12	0.15	0.02	0.43	0.03	0.04	4.36
HD13	NT	NT	NT	NT	NT	NT
HD14	0.10	0.01	0.09	0.11	0.01	3.07
HD15	0.54	0.02	0.16	0.16	0.03	2.62
HD16	0.11	0.01	0.03	0.09	0.01	0.72
HD17	0.91	0.06	0.27	0.24	0.18	0.47
HD18	NT	NT	NT	NT	NT	NT
HD19	NT	NT	NT	NT	NT	NT
HD20	NT	NT	NT	NT	NT	NT
HD21	0.20	0.14	0.03	0.23	0.06	1.99
HD22	NT	NT	NT	NT	NT	NT
HD23	NT	NT	NT	NT	NT	NT
HD24	0.32	0.02	1.93	0.02	0.01	0.92
HD25	NT	NT	NT	NT	NT	NT
HD26	NT	NT	NT	NT	NT	NT
HD27	NT	NT	NT	NT	NT	NT
HD28	NT	NT	NT	NT	NT	NT
HD29	NT	NT	NT	NT	NT	NT
HD30	NT	NT	NT	NT	NT	NT
HD32	0.01	0.58	0.29	0.01	0.01	2.68

*Values indicated the percentages of CD8 T cells producing IFN-γ. **Unstimulated PBMC served as a negative control. ***Stimulated with Concanavalin A served as a positive control.

Table 8.3 Total data for CD8 T cell responses to HCMV antigens by intracellular cytokine staining for TNF-α*

Donor	IE1	IE2	pp65	gB	Neg**	Pos***
HD01	0.55	0.06	0.16	0.10	0.03	4.48
HD02	0.09	0.10	0.33	0.19	0.02	2.68
HD03	1.94	0.06	0.15	0.09	0.03	6.65
HD04	0.19	0.06	0.02	0.07	0.06	2.75
HD05	3.91	0.04	0.86	0.07	0.03	5.14
HD06	2.54	0.05	0.68	0.25	0.06	4.86
HD07	3.87	0.32	0.79	0.15	0.06	3.77
HD08	0.49	0.16	0.28	0.30	0.07	3.37
HD09	8.13	0.04	0.08	0.05	0.02	0.73
HD10	NT	NT	NT	NT	NT	NT
HD11	3.40	0.04	0.32	0.11	0.02	1.14
HD12	0.03	0.03	0.17	0.04	0.03	4.25
HD13	NT	NT	NT	NT	NT	NT
HD14	0.05	0.08	0.05	0.27	0.05	1.21
HD15	0.65	0.08	0.14	0.16	0.06	1.56
HD16	0.06	0.03	0.05	0.05	0.03	0.35
HD17	0.87	0.00	0.19	0.11	0.09	0.28
HD18	NT	NT	NT	NT	NT	NT
HD19	NT	NT	NT	NT	NT	NT
HD20	NT	NT	NT	NT	NT	NT
HD21	0.14	0.37	0.13	0.15	0.03	1.33
HD22	NT	NT	NT	NT	NT	NT
HD23	NT	NT	NT	NT	NT	NT
HD24	0.42	0.00	1.08	0.05	0.02	0.86
HD25	NT	NT	NT	NT	NT	NT
HD26	NT	NT	NT	NT	NT	NT
HD27	NT	NT	NT	NT	NT	NT
HD28	NT	NT	NT	NT	NT	NT
HD29	NT	NT	NT	NT	NT	NT
HD30	NT	NT	NT	NT	NT	NT

*Values indicated the percentages of CD8 T cells producing IFN-γ. **Unstimulated PBMC served as a negative control. ***Stimulated with Concanavalin A served as a positive control.

Table 8.4 List of antibodies/chemicals/reagents/consumables

A. Antibodies/kits	Manufacturer	Catalogue no
Human IFN-γ ELISpot Kit	Mabtech, UK	3420-2A
Mouse Anti-human CD3	BD Biosciences, UK	552127
Mouse Anti-human CD4 APC	BD Biosciences, UK	555349
Mouse Anti-human CD4 PerCP-Cy5.5	BD Biosciences, UK	560650
Mouse Anti-human CD8 APC	BD Biosciences, UK	555369
Mouse Anti-human CD8 FITC	DAKO, UK	45-0073
Mouse Anti-human CD8 PE	DAKO, UK	45-0037
Mouse Anti-human CD8 PerCP-Cy5.5	BD Biosciences, UK	560662
Mouse Anti-human HHV-6B	Millipore, USA	MAB8535
Mouse Anti-human IFN-γ FITC	BD Biosciences, UK	340449
Mouse Anti-human IFN-γ PE	BD Biosciences, UK	560742
Mouse Anti-human TNF-α	BD Biosciences, UK	554513
Ready set go ELISA kit Human IFN-γ	eBioscience, UK	88-7316-88

B. Chemicals/reagents	Manufacturer	Catalogue no.
Amphotericin B	Sigma Aldrich, UK	A2942
Bovine serum albumin (BSA)	Sigma Aldrich, UK	A8327
Brefeldin A	eBioscience, UK	00-4506-51
Celltrace CFSE cell proliferation kit	Molecular Probe, USA	C34554
Cytofix fixation buffer	BD Biosciences, UK	51-9006613
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, UK	D2650
Dulbecco's phosphate buffered saline	Sigma Aldrich, UK	D5652
Ficoll-paque premium	GE Healthcare Life Sciences, UK	17-5442-03
Foetal bovine serum (FBS)	Sigma Aldrich, UK	F7524
Golgistop protein transport inhibitor (Monensin)	BD Biosciences, UK	51-2092KZ
Human AB serum	Sigma Aldrich, UK	H4522
Heparin sodium (mucous injection BP)	LEO Pharma, UK	DE0248
IC fixation buffer	eBioscience, UK	00-8222-49
L-glutamine	Sigma Aldrich, UK	G7513
Penicillin/streptomycin	Sigma Aldrich, UK	P0781
Permeabilisation buffer (10x)	eBioscience, UK	00-8333
RPMI 1640 media	Sigma Aldrich, UK	R5886
Trypan Blue	Sigma Aldrich, UK	T-8154
Tween-20	Sigma Aldrich, UK	P1379
Vectasheld (mounting medium with DAPI)	Vector, UK	P1203

C. Consumables	Manufacturer	Catalogue No.
ELISA plates (96 well, flat, high protein binding EIA/RIA plate)	Corning, USA via Appleton Woods, UK	CC679
ELISpot plates (Multiscreen ^{HTS} filter, 0.45µm hydrophobic, high protein binding membrane)	Millipore, USA	MAIPN45
MACS cell separation LD columns	Myltenyi Biotech Ltd, Germany	130-042-901
Microplate 24-Well with lid	Sarstedt, USA	82.1583
Microtest Plate 96-Well Vee Bottom	Iwaki, Japan	3820-024
Transfer pipette (3.5ml sterile)	Sarstedt, USA	86.1171.001